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ARBUSCULAR MYCORRHIZAL FUNGI OF NORTHERN WHITE CEDAR (Thuja occidentalis L.): HABITAT EFFECTS ON FUNGAL COMMUNITIES AND INOCULUM EFFECTS ON PLANT GROWTH ON ACID PEAT SOILS

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Table of Contents

Preface

Chapter 1, Effects of mycorrhizal inoculation, fertilization, and liming on growth and nutrient acquisition of *Thuja occidentalis* L. seedlings on acidic peat soil; in preparation for submission to publish in a peer-reviewed journal. Guswarni Anwar conducted and designed the study, collected and analyzed the data, and wrote the manuscript. Dr. Erik A. Lilleskov and Dr. Rodney A. Chimner contributed to experimental design and edited the manuscript.

Chapter 2, Effect of arbuscular mycorrhizal inoculum, AM host proximity, and other environmental factors on growth and survival of *Thuja occidentalis* seedlings in a poor fen; in preparation for submission to a peer-reviewed journal. Guswarni Anwar conducted and designed the study, collected and analyzed the data, and wrote the manuscript. Dr. Erik A. Lilleskov and Dr. Rodney A. Chimner contributed to experimental design and to editing the manuscript.

Chapter 3, Structure and composition of arbuscular mycorrhizal community on *Thuja occidentalis* roots in peatland, mesic upland, and mine tailing habitat types; in preparation for submission to a peer-reviewed journal. Guswarni Anwar conducted and designed the study, collected the data, and wrote the manuscript. Dr. Louis J. Lamit performed the bioinformatic analysis and contributed to analysis of the data and wrote the bioinformatic methods. Dr. Erik A. Lilleskov and Dr. Rodney A. Chimner contributed to experimental design and to editing the manuscript.

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Abstract

The relationship of arbuscular mycorrhizal (AM) fungi with northern white cedar (NWC) was examined from the perspective of both fundamental questions about habitat specificity in the root fungal community, as well as applied questions regarding AM fungal efficacy in NWC restoration in peat soils. I performed two experiments testing the effects of AM fungi on survival, growth, and nutrition of NWC seedlings; and one molecular study to determine the habitat effects on community composition of NWC root-associated fungi. First, a greenhouse AM inoculation experiment was conducted in factorial combination with fertilization and liming to examine conditional effectiveness of AM fungal inoculation. Second, a field experiment in a poor fen was conducted to determine effectiveness of AM fungal inoculation, AM plant proximity, and environmental factors on survival, growth, and nutrition of NWC seedlings. Third, an observational study employed Illumina sequencing to determine habitat effects on diversity and composition of NWC root-associated fungal communities in mine tailings, peatlands, and uplands. AM inoculation of NWC had different outcomes in the greenhouse and field experiments. In the greenhouse AM fungi significantly increased all plant growth and many nutrient metrics, whereas in the field there were no significant inoculum effects. This might be due to the differences in several experimental conditions. Seedlings in the greenhouse grew under high environmental control, higher pH, using commercial inoculum, and with no competition. In contrast, the field experiment was conducted without environmental controls, with native inoculum under more acidic and competitive conditions. However, in addition to pH and light effects, we observed positive AM plant proximity effects on growth and nutrition, perhaps indicating a mycorrhizal role in NWC seedling success in poor fens. In the fungal community

analysis, unidentified Glomeraceae were the dominant AM fungi across all habitats. Total fungal and AM fungal community richness was higher in bog and upland than in stamp sands. Fungal community composition within Glomeromycota and all fungal taxa were both significantly different between the mine tailing and the other two habitats. There were taxa with both broad and narrow habitat associations that are potential targets for general vs habitat-specific AM inoculum.

Summary

Arbuscular mycorrhiza (AM) fungi colonize most terrestrial plant species and some wetland plants. These fungi assist plant growth and nutrition especially phosphorus mobilization in nutrient-poor soils. Therefore, AM fungi are an effective tool of restoration and reclamation projects in degraded lands, in both peatlands and uplands. In many cases, degraded lands exhibit reduced productivity due to mineral nutrient deficiency, soil drought, and increased heavy metals (soil toxicity). Such conditions potentially reduce or eliminate indigenous AM propagules. It is important to reintroduce AM fungi into the disturbed lands to support plant growth and accelerate restoration and reclamation programs. However, relatively little is known about AM fungi role in the establishment and growth of northern white cedar (*Thuja occidentalis* L.).

Northern-white cedar is an arbuscular mycorrhizal tree species that is common in the northeastern United States and Canada. This species occurs in both upland and wetland habitats. In wetlands it is predominantly found in rich swamps (forested rich fens) on soils with slightly acidic (5.5) to neutral pH; but also occurs in more acidic peatlands. Northern-white cedar provides a variety of benefits, particularly related to wood products and wildlife habitat. In recent years, NWC have been negatively affected by white-tailed deer browsing, harvesting, low recruitment, and high competition with associated trees and shrubs.

Fens are a globally important peatland type, including in the northern Great Lakes region. At the most acidic and nutrient-poor end of the fen continuum (poor fens), not many tree species are able to occupy this habitat. Poor fens are predominantly covered by ericaceous shrubs and sphagnum mosses. Acidity, high water tables, and low nutrient availability cause these ecosystems to support low

tree productivity. When these ecosystems are disturbed they can be subject to restoration or mitigation programs that include planting major trees such as Pinaceae and NWC. Success of these restoration efforts is variable, perhaps because the role of mycorrhizal fungi has not been considered as part of these efforts.

The main goal of our study was to improve understanding of the relationship of AM fungi with NWC. We approached this from the perspective of both basic questions about the community of fungi involved in the symbiosis in different habitats, as well as applied questions regarding the efficacy of AM fungi in improving success of NWC restoration in peat soils. This was conducted using two experimental series looking at the effects of AM fungi on survival and growth of NWC seedlings; and one molecular study to determine occurrence of AM fungi in association with NWC in three contrasting habitat types. To achieve this goal we had three primary objectives and associated activities.

The first objective was to determine efficacy of AM fungi to improve growth and nutrient acquisition of NWC in peat soils, as a low impact approach to peatland restoration without chemical additions (e.g., fertilization and liming). We conducted an AM inoculation greenhouse experiment in factorial combination with fertilization and liming to examine effectiveness of AM fungal inoculation under a range of environmental conditions. We also determined success of AM fungal colonization by quantifying occurrence of their structures in NWC roots. We germinated NWC seeds and treated the seedlings with commercial AM fungi inoculum, fertilization, and liming. We measured height, diameter, plant biomass, and nutrient acquisition (N, P, Cu). Our findings showed AM inoculation without fertilization significantly increased all growth and nutrient metrics of the seedlings except N and Cu concentration. The positive impact of AM inoculation on plant growth and nutrient acquisition was similar to fertilizer. Our study showed liming alone did not improve NWC growth and

nutrient acquisition. Fertilization, and to a lesser extent liming, reduced the efficacy of AM inoculum to improve plant growth and nutrient acquisition. We conclude that using AM inoculation alone effectively increased NWC growth and nutrient supply and reduced the need for fertilizer and lime in peatlands. AM inoculation could be an ecologically and economically favorable alternative to enhance the success of restoration of NWC in acid peatlands.

The second objective of our study was to determine effectiveness of AM fungal inoculation, AM plant proximity, and environmental factors on survival, growth, and nutrient uptake of NWC seedlings in a field study in a poor fen. We assessed whether native AM fungal inoculum, AM plant proximity, AM plant index (ordination of basal area and percentage of vegetation cover for the major mycorrhizal types), and other environmental factors (soil pH, water table depth, peat bulk density, and light intensity) affected NWC survival and growth in a poor fen. We conducted the experiment in a poor fen dominated by ericaceous shrubs and sphagnum mosses with patchy distribution of ectomycorrhizal (ECM) and AM trees. We planted 396 NWC seedlings along 70-100 m long transects parallel to the peat margin located 10, 50, 100, 150 and 200 m from the peatland margin. We randomly established plots within each 5 m interval over the length of these transects. We placed four points within each plot in a crossed design with each axis 2 m in length. At planting, half of the seedlings were inoculated with native AM inoculum using fresh NWC fine roots taken from the study area, and half were left as un-inoculated control treatments. We applied a range of statistical analyses to determine treatment effects on NWC growth and nutrient acquisition. After 12 months from the initial planting, we found that AM inoculum had no significant effect on survival, plant growth, and nutrient acquisition (N, P, Ca) whether analyzed alone or in interaction with other environmental factors. Light was the only significant predictor of survival,

with greater survival under higher light. AMF plant proximity significantly affected plant growth and nutrient acquisition, with NWC seedling growth and nutrient supply higher when closer to AM trees. Relationship of AM plant proximity with light and ERM (ericoid mycorrhizal) plants were significant, with lower light and Ericaceae cover near AM trees. We conclude that AM inoculant was not able to improve survival rate, growth and plant nutrition. However, the fact that AM plant proximity significantly increased the growth and nutrient supply indicates the need for further analysis to test whether enhanced AM colonization or other factors such as reduced competition from Ericaceae are the cause.

The third objective of our study was to determine effect of habitat type on diversity and composition of NWC root-associated fungal communities in three habitat types (peatland, mine tailings, upland), and to determine the effect of environmental factors and plant community as predictors of fungal community composition and structure. We assessed AM fungi that belong to the phylum Glomeromycota as predominant fungal species in all the habitats. We conducted a molecular study with DNA based next generation (Illumina) sequencing to identify structure and composition of fungal species especially Glomeromycota in the three habitats. We collected NWC root samples in each habitat type (14 sampling locations: 5 peatland, 3 stamp sand, 6 upland), and measured soil and foliar chemistry. We extracted DNA from the root samples to be used in high-throughput sequencing with the Illumina MiSeq. The resulting sequences were subjected to bioinformatics pipeline to cluster sequences into operational taxonomic units (OTUs). We statistically analyzed the data using PERMANOVA to test the effect of habitat on fungal community similarity. We also used non-metric multidimensional scaling (NMDS) to test habitat effects on the OTU composition. We determined indicator species from each habitat type and tested the effect of habitat, soil pH, and plant

community on rarefied OTU richness and evenness. Our finding showed Glomeromycota, mostly in the Glomeraceae, were common members of the fungal community across the habitats. Fungal community richness for all taxa and for Glomeromycota was significantly affected by habitat type. Stamp sands had the lowest richness across the habitats. Unidentified Glomeraceae OTUs were the most abundant Glomeromycota in this study. Fungal community composition within Glomeromycota and all fungal taxa were significantly affected by habitat type, perhaps mediated by differences in pH and AM plant index. Considering the consistent occurrence of Glomeraceae in the three habitats, this family may be the source of important inoculant in seedling planting program of land restoration projects.

In conclusion, AM inoculation on NWC in poor fen soils showed different results between the greenhouse and field experiment. The greenhouse experiment showed AM fungi significantly increased all plant growth and nutrient metrics. In contrast, the field experiment showed non-significant effects. This might be due to the differences in several experimental conditions. Seedlings in the greenhouse grew under high control of environmental factors, higher pH, with commercial inoculum, and no competition with other plants. In contrast, the field experiment was conducted without environmental controls, with native inoculum, under more acidic conditions, and the seedlings faced high competition with neighboring plants. However, our positive AM plant proximity effects also suggest that there might be a mycorrhizal role in NWC seedling success in these habitats. Our positive growth and nutrition responses suggest it is possible to use AM fungi as an inoculum on NWC seedlings in restoration projects, at least on mildly acidic to circumneutral peat. More tests should be carried out on best practices in inoculation under field conditions, and to distinguish mycorrhizal from other influences on seedling success. Moreover,

dominance of different AM OTUs in mine soils vs peatland and upland habitats suggests that inoculum sources with both narrow and broad habitat ranges are available, and should be tested for efficacy over a broad range of site conditions.

Chapter 1. Effects of mycorrhizal inoculation, fertilization, and liming on growth and nutrient acquisition of *Thuja occidentalis* L. seedlings on acidic peat soil[1]

1.1. Abstract

Arbuscular mycorrhizal (AM) fungi are hypothesized to assist growth of northern white cedar (NWC) in acid peatlands, yet there is little direct evidence that they can provide sufficient resources, especially nitrogen, from unfertilized peat soils. Our objective was to determine mycorrhizal efficacy to support NWC growth and nutrient supply as part of a low-impact approach for ecological restoration of NWC in oligotrophic peatlands. We tested the effectiveness of AM inoculation in a greenhouse experiment in factorial combination with fertilization and liming. We also determined AM colonization rate in the different treatment combinations. We found that AM inoculation in the absence of fertilization significantly increased all growth and nutrient concentrations and content variables of the seedlings, except N and Cu concentration. Fertilizer alone had a similar impact on plant growth and nutrient acquisition when compared to unfertilized AM inoculation treatments. We also found that liming alone was ineffective at increasing NWC growth and nutrient uptake. There were many interactions of AM inoculation with liming and fertilization. Specifically, the effect of AM inoculation on many growth and nutrition metrics was reduced in the presence of both fertilization and liming. We conclude that using AM inoculation alone was able to improve NWC growth and its nutrient acquisition and reduce the need for fertilizer and lime in peatlands.

[1] The material contained in this chapter is in preparation for submission to a journal.

1.2. Introduction

Northern white cedar (*Thuja occidentalis* L; NWC) is a dominant tree in forested wetlands in northeastern North America. It is most prominent in rich swamps or forested rich fens with pH ranging from 5.5 to 7.2 (Johnston, 1990; Fraver et al., 2009), however, is also found in acidic peatlands (poor fens of pH ~ 4.5).

Arbuscular mycorrhizal (AM) fungi colonize NWC (Brundrett et al., 1989, Matthes-Sears et al., 1992; Bainard et al., 2011) and may assist NWC colonization of these nutrient-poor soils by enhancing nutrient, especially P uptake. However, AM fungi are relatively uncommon in acidic peatlands. It is largely unknown whether arbuscular mycorrhizal fungi can play an important nutritional role for NWC colonization in these acidic peatlands. Understanding the role of AM fungi on NWC is important not only for expanding fundamental knowledge of AM fungi in peatlands, but also because NWC is the subject of intensive restoration efforts (e.g., Kangas et al., 2015, Palik et al., 2015). Therefore, it is necessary to develop economically feasible and environmentally sound techniques to enhance NWC ability to establish and grow in these marginal sites.

Historically, both fertilization and liming have been commonly used to improve seedling growth in marginal soils during restoration (Moore, 2000; Walker, 2002; Jonard et al., 2010; Pabian et al., 2012) However, fertilization and liming can be costly and lead to accumulation of heavy metals in soils, aquatic ecosystems, and plants (Braekke, 1999, Savci, 2012; van der Ent et al., 2013, Marchand et al., 2014).

Mycorrhizal inoculation effectively reduced fertilizer (N and P) use by enhancing capability of host plants to take up both nutrients from soil (Singh, 1998, Tawaraya et al., 2007). However, Hoeksema et al. (2015) found that AM fungi

effectively overcame P limitation but not N limitation. Given that acid peatlands are commonly N limited, it is important to understand whether AM inoculation can reduce the need for fertilizer and lime additions in peatlands.

Both fertilization and liming could also reduce the efficacy and abundance of mycorrhizal fungi (Treseder, 2004). Fertilizers, through enhancing N and P, generally eliminate nutritional benefits of mycorrhizas (Nijjer et al., 2010), and instead can potentially turn mycorrhizal symbiosis into a parasitic rather than mutualistic relationship (Johnson, 1993). A mutualism commonly occurs in high N and low P, and vice versa for parasitism symbiosis (Johnson et al., 2014). Liming could also decrease the benefit of AM fungi because increasing pH from acid pH to about pH 7, increases availability of inorganic P (Schlesinger, 1997), and reduces amount of fungi (Ivarson, 1977). However, at higher pH, P availability declines again and so mycorrhizal colonization and benefits could increase (Anderson et al., 1996; Borja and Nielsen, 2008).

Copper availability in peatlands or organic soils is generally low and can limit plant growth (Rehm, 2002). Cu uptake by AM fungi might be affected by availability of soil P, where their activity will be hampered under high P concentration (P fertilizer), which likely reduces absorption of Cu by plants (Lambert et al., 1979). However, their role in Cu acquisition is still unclear (Leyval et al., 1997). Some studies found increased Cu absorption by AM plants (Killham and Firestone, 1983; Weissenhorn and Leyval, 1995) whereas others showed the reverse (Leyval et al., 1991).

Given the paucity of information of the effect of AM fungi on NWC seedling growth and macro- and micronutrient uptake on poor peat soils, our objective was to fill this gap. The aims of the present greenhouse study were to 1) test if AM inoculation increased growth of NWC seedling on oligotrophic peat soil, and 2)

quantify if fertilization and liming of the soil modified results of inoculation. We hypothesized that 1) AM inoculation would increase NWC growth and nutrient acquisition, especially P, and 2) the positive impact of AM inoculation of NWC would be reduced in fertilized and limed seedlings.

1.3. Materials and methods

1.3.1. Study site

A greenhouse study was conducted at the School of Forest Resources and Environmental Science, Michigan Technological University. Seeds of NWC were obtained from the USDA Forest Service (J.W. Toumey Nursery, Watersmeet, MI). The peat soil (pH 4.4) used for this study was obtained from a forested poor fen near Painesdale, Houghton County, MI (N 47.01349°, W 88.43082°). The peatland is dominated by non-mycorrhizal mosses (*Sphagnum* and *Polytrichum)*, dwarf shrubs belonging to Ericaceae colonized by ericoid mycorrhizal fungi, and trees dominated by black spruce and tamarack colonized by ectomycorrhizal fungi. To avoid contamination with native inoculum from sparsely distributed AM hosts, soil was collected under Ericaceae from an area with no NWC or other AM host species present within 100 meters.

1.3.2. Experimental treatments

Prior to sowing, NWC seeds were soaked overnight in cold water. The seeds were germinated on flats filled with a mixture of pasteurized (70°C) vermiculite (Sunshine Vermicullite, Sun Gro Horticulture Canada Ltd) and potting soil (Sunshine Mix 1, Sun Gro Horticulture Canada Ltd) with a 1:2 ratio. The seeded flats were placed within a mist chamber in the greenhouse for approximately two months until the seeds germinated and grew to an average height of 2 cm.

The experiment was a full-factorial completely random experimental design consisting of three factors: mycorrhizal inoculation (M), fertilization (F), and liming (L), each with two levels (with and without the factor). Each treatment combination was replicated ten times. We used Osmocote Plus 15-9-12 (N-P-K) slow release fertilizer that included some micronutrients such as magnesium, sulfur, boron, copper, iron, manganese, molybdenum, and zinc (Everris NA, Inc., Dublin, OH). For lime, we used garden and lawn lime (Mayfille Limestone Inc, Mayfille, WI) consisting of 22% calcium (Ca) and 12% of magnesium (Mg). Fertilizer and lime were mixed with the soils about a month prior to the initiation of the experiment, applied based on their manufacture's recommendation, with dosage 1.65 g fertilizer/500 ml soil and 1.15 g lime/500 ml soil. Arbuscular mycorrhizal inoculum consisted of *Rhizophagus intraradices*, *Glomus mosseae*, *G. aggregatum*, and *Claroideoglomus etunicatum* (*Tri-C Enterprises, Chino, CA*) that contained 120 propagules/cc. Control inoculum material for other effects of inoculum was pasteurized at 70°C. Mycorrhizal inoculum was applied in the growing media when the seedlings were transplanted. The inoculum was placed around seedling roots at the rate of 3.4 g per seedling as recommended by the manufacturer. On un-inoculated treatments, the seedlings were given the same amount of pasteurized AM inoculum. On March 12, 2014 the previously germinated seedlings were transplanted into Deepots 7 cm in diameter by 25 cm tall (Stuewe and Sons, Inc. Tangent, OR) containing 500 ml unsterilized- field collected peat soils treated as described above. The pots were randomly arranged in racks on greenhouse benches. Day length was set at 16 hours with supplemental lighting via Halco metal halide lamps (Prolume MP 400/BU), and temperature maintained at 22°-24°C. Seedlings were watered daily using tap wat

1.3.3. Data collection

As a non-destructive measure of the effect of the treatments on growth, seedling height was measured monthly. Seedlings were harvested after 11 months from their transplanting time (February 7, 2015). At harvest, height and diameter of seedlings were measured. In addition, shoots and roots of the seedling were separated. Roots were washed with tap water, 0.3 g (wet weight) root subsamples were taken for assessment of mycorrhizal colonization (see below), then residual roots and shoots were placed into paper bags and oven dried (65^{o} C) until their weights were constant. After drying, we measured root and shoot dry weight.

Healthy fine roots to be used in measurement of mycorrhizal colonization were subsampled from around the root collar where new roots emerged. To measure the effectiveness of mycorrhizal inoculum, we first cleared and stained the roots following the protocol of Vierheilig et al. (2005). Briefly, this entailed clearing the roots by submerging them in 30 ml 10% KOH solutions and placing them in a water bath at 90^{o}C. When KOH solution became colored, the solution was changed until it remained clear. Cleared roots were rinsed with DI water and stained overnight with the staining solution with concentration 0.06% Chlorazol E Black (CEB, Acros Organic (0.3 g), lactic acid (100 ml), glycerol (200 ml), and DI water (200 ml). Finally, the roots were rinsed with DI water and placed in destaining solution consisting of lactic acid (200 ml), glycerol (100 ml), and DI water (400 ml). The destaining solution was changed until solution remained clear. Roots were mounted on slides in PVLG gel (a mixed solution of DI water (100 ml), lactic acid (100 ml), glycerol (100 ml), and polyvinyl alcohol (16.6 g) (van Diepen, 2008). Next we scored the percentage of fungal colonization on the stained roots under the microscope, based on presence of AM and other fungal structures including aseptate AM hyphae, septate non-AM

hyphae, arbuscules, coils, and vesicles (van Diepen, 2008). We measured the percentage of colonized roots under 200x magnification, with a total of 100 root transects per slide. Photos of mycorrhizal structure on colonized roots were taken using a microscope-mounted 5.0 megapixel digital camera (Leica DFC480, Cambridge, UK).

We measured leaf nutrient concentration and content (N, P, C, and Cu) in dried NWC leaves. The leaves were ground use a mortar and pestle, and analyzed at Laboratory of Forest Ecology Stable Isotope, SFRES, at Michigan Tech. For %C and %N we used a Costech 4010 elemental analyzer (Costech Analytical Technologies Inc, Valencia, CA, USA) calibrated with atropine. For %P and %Cu, we used inductively coupled plasma optical emission spectrometry on a Perkin Elmer Optima 7000DV ICP-OES (PerkinElmer Inc, Waltham, MA, USA) using the dry ash method (Miller 1998). Foliar nutrient content was derived from dry mass and concentration data. To determine the efficacy of the liming treatment, we measured soil pH of each treatment at the termination of the experiment on pooled, 2 mm sieved soils. We measured soil pH use a pH conductivity meter (Denver Instrument Model 220, Denver Instrument, Arcada, CO, USA).

1.3.4. Data analysis

The effect of treatment factors on NWC growth metrics (height, diameter, biomass, and biomass allocation) and nutrient status (concentration and content of N, P, and Cu; N:P), and percentage of mycorrhizal colonization were statistically tested using SAS program (SAS Institute Inc., Cary, NC, USA) using generalized linear models. We accounted for lack of normality using transformations when needed, and lack of homogeneity of variance was accounted for using an appropriate "group" term that permitted analysis under heterogeneous variance. There was no

transformation needed for the variables height, total biomass, P and Cu content, %Cu, and NP ratio. We transformed other variables as following: square root (diameter, root biomass, root shoot ratio, N content) and log 10 (shoot biomass, %N, %P).

1.4. Results

1.4.1. AM structures presence

AM fungal structures such as aseptate hyphae, vesicles, and arbuscules were more abundant in inoculated than uninoculated treatments (Table 1.1). The most common structures were aseptate AM hyphae, which appeared in all inoculated treatments and no inoculated treatments. Few arbuscules were observed in this experiment, and those that were observed appeared to be degraded. Vesicles were also found in limited number. Septate hyphae, indicating non-mycorrhizal root endophytes, were highest in the fertilized + inoculated treatment combination (Fig 1.1).

1.4.2. Seedling Growth

For virtually all growth metrics, there was a significant and roughly equivalent positive effect of both fertilization and inoculation. However, these effects were mostly non- additive: the positive effect of both treatments was much stronger alone than when in combination, resulting in many significant treatment interactions (Table 1.2; Fig 1.4; Fig. 1.8).

In comparison with the other two treatments, the liming main effects were weaker, and its interactions with inoculation and fertilization differed. There were significant negative main effects of liming on shoot and total biomass. In the presence of fertilization, the negative effects of liming were reversed, leading to

significant interactions for root biomass, shoot biomass, and total biomass (Table 1.2; Fig 1.4; Fig 1.6). In contrast, the negative effects of liming were enhanced in the presence of mycorrhizal inoculation, leading to large reductions in root, shoot and total biomass in the mycorrhizal limed treatment relative to the mycorrhizal un-limed treatment, which manifested as significant liming x inoculation interactions for root biomass and total biomass (Table 1.2; Fig 1.4; Fig 1.7).

1.4.3. Nutrient acquisition

For almost all nutrient metrics, both fertilization and mycorrhizal inoculation showed the same positive effects, except on %N and %Cu. When in combination, fertilization and inoculation had a smaller or no additive effect, leading to significant fertilization x inoculation interactions for all variables except %P (Table 1.3; Fig 1.4; Fig 1.8).

Liming showed different effects from both fertilization and inoculation. There were significant negative main effects of liming on %P, P content, and N content, and significant positive main effects of liming on NP ratio. Liming had a weak significant interaction with inoculation for both %N and %P (Table 1.3; Fig 1.5; Fig 1.8). In both cases this resulted from a positive effect of inoculation in combination with liming vs. negative (%N) or non-significant (%P) effect in the absence of liming.

1.5. Discussion

1.5.1. Mycorrhizal inoculation effects on growth and nutrient acquisition

Overall, eleven months after treating the NWC seedlings with the experimental treatments, AM inoculation positively affected all growth metrics of NWC seedlings and nutrient measurements except N concentration. These results supported our hypothesis that on unfertilized acid peat soil AM fungi inoculation was

able to improve NWC growth and nutrient supply, especially P, reducing the need for fertilizer and liming. Our results also show that AM inoculum that consisted of fungi of *Rhizophagus intraradices*, *Glomus mosseae*, G. *aggregatum*, and *Claroideoglomus etunicatum,* successfully colonized NWC seedlings.

Many studies have exhibited that benefits of AM fungi for plants are predominantly obtained in sites with limited nutrients, especially P (Liu et al., 2000; Tawaraya, 2003; Smith & Read, 2008; Smith & Smith, 2011). This is consistent with their importance in enhancing plant P uptake, whereas their role in N uptake is less clear (Smith and Smith, 2011). Our study shows that mycorrhizal growth response (MGR) was very strong, positive and significantly greater on inoculated seedlings (M) than that of on uninoculated seedlings (control). Likewise, we found mycorrhizal inoculation resulted in positive effects to plant growth in absence of fertilization and liming. Even, these treatments hindered efficacy of AM fungi (Fig 1.4; Fig 1.5; Fig 1.7, Fig 1.8).

Enhanced plant growth was likely caused by increasing nutrient availability especially P. Analysis of foliar N : P ratio shows that mycorrhizal inoculation was able to reduce the ratio, which is a good indicator of increasing P availability to the plants (P concentration and content). These results suggest that availability of the limiting resource (P) as a major driver to control plant growth with an assumption that other resources were not limiting. Liebig's law of the minimum state that plant growth is predominantly determined by the most limiting resource, although plant growth is mostly controlled by co-limited factors by multiple resources (Harpole et al., 2011; Johnson et al., 2014). Although AM fungi are capable of N acquisition from either inorganic or organic forms (Smith and Smith, 2011), many studies report that AM fungi do not increase N availability as much as P availability (Liu et al., 2000; Valentine et al., 2001; Jin et al., 2012). In acid peatlands, where N is often limiting

(Bayley et al., 2005) and other species have specific adaptations to increase N uptake (Smith and Read, 2008), this could reduce the efficacy of AMF of NWC relative to competitors. Tissue N concentration, P concentration, and N:P ratios in our study reveal that mycorrhizal inoculation successfully alleviated P deficiency, but not N limitation (Fig 1.6). Tissue N:P >16 indicates P limitation (Koerselman and Meuleman, 1996; Johnson et al., 2014), where AM fungi act as a mutualistic symbiont where they supply surplus P for plant photosynthate. Meanwhile, tissue N:P <14 depicts limitation in N availability where AM fungi may act as commensal or parasitic symbiont (Johnson et al., 2014). Under N-limited systems, AM fungi may be incapable of providing N surplus for host plants since their N demand (per unit biomass) is higher than their hosts (Johnson et al., 2014; Hodge & Storer, 2015). It is assumed that AM fungi used N to fulfill their own nutritional needs before supplying it to host plants. Hodge and Fitter (2010) found that the AM extraradical hyphae had N concentration seven- to ten-fold higher than that of plant shoots and roots. Under limited soil N availability, AM fungi cannot supply N to their hosts (Hoeksema et al., 2015). In addition, in pot experiments AM external hyphae and plant root systems have to compete using the same soil volume so it is less likely AM external hyphae would explore different soil resources (Hodge, 2000; Hodge, 2001).

Our findings indicate that AM fungi reduced foliar Cu concentration, although all treatments were several-fold above the deficiency threshold of ~4 ppm for other conifers (Schmitt & White 1988). Reduction of Cu concentration in the roots and shoots has been reported by several authors (Zhang et al., 2009; Latef, 2011 ; Meier et al., 2015). It is possible that reduction of copper concentration in the plants is related to AM fungi benefits in protecting the plants from Cu toxicity and increasing P availability. Latef (2011) suggested AM plants had protection from Cu exposure by enhancing availability of phosphorus and improving plant growth. Timmer and

Leyden (1980) reported there was negative correlation between P availability and Cu uptake by plants, where increasing P supply led to diminished copper acquisition by plants. In addition, increasing copper uptake is also influenced by nitrogen where a soluble organic N will associate with Cu compound to translocate copper throughout the plant from the xylem and phloem saps (Singh and Swarup, 1982). Hence, if reduction of N uptake occurred, it would affect alleviation of copper uptake. Nevertheless, mechanisms of AM fungi in Cu accumulation have not still been clear. Glomalin produced by AM fungi could be a major consideration in decreasing Cu concentration where this glycoprotein can support sequestration of Cu and other heavy metals (Gil-Gardeza et al., 2014). Several studies about copper-binding capacity of AM hypae revealed that AM fungi become a biological barrier in Cu translocation in plant tissues where glomalin will prevent Cu transfer from the roots to shoots (Joner and Leyval., 1997; Joner et al., 2000; Toler et al., 2005; Zhang et al., 2009).

Presence of AM fungi structures such as arbuscules and vesicles mirrors AM colonization in the roots, although presence of hypha alone can be evidence of their association with the roots. Our study showed limited arbuscules and vesicles in AM-colonized roots. We assumed that it was affected by soil pH status. Duke et al. (1994) suggested that lack of arbuscules might indicates plant roots are in nutrient-rich conditions and the plant is less responsive to P supply by AM fungi. Abbot et al. (1984) and Braunberger et al. (1991) stated that proportion of arbuscules to vesicles might be used to understand relative benefit of mycorrhizal fungi to the plant. However, Brundrett and Kendrick (1988) suggested that arbuscules are ephemeral structures, sometimes they are not present in the samples particularly when the sample roots are collected in active forms.

1.5.2. Interaction of mycorrhizal inoculation with fertilization and liming

Fertilization and liming have generally been applied to increase plant growth, improve soil fertility, and reduce soil acidity in peatland restoration (Huotari et al., 2007; Bjork et al., 2010; Caporn et al., 2007). Their ability to supply some essential nutrients and increase soil pH in short term are the major consideration to use them to restore the degraded peatlands, even though there are economic and ecological costs of these practices. Both of these practices appear to reduce plant response to mycorrhizal inoculation. Our study showed benefits of inoculation are greatest in the absence of fertilization (Fig 1.7; Fig 1.8). These results indicate that mycorrhizal inoculation is an important alternative to fertilization of NWC, and might be beneficial under liming to a high soil pH.

In conclusion, AM inoculation successfully improved nutrient status and growth of NWC seedlings in acidic peat soils, with benefits similar to those of fertilization. This indicates that AM fungi might be an alternative to enhance success of NWC restoration projects without the need for additional liming and fertilization. AM inoculation would sustain in the plant roots under the favor environments. However, two factors might limit our ability to infer success in acid peatland soil. Firstly, our study was a greenhouse experiment, hence eliminating plant competition. Presence of other plants might restrict NWC growth because all the plants require the same basic factors to support their growth such as nutrient, light, water, space, and other factors, and other species have adaptations that might favor them for competition for limited N (Johnston, 1990; Weber et al., 2005). Secondly, AM efficacy is determined by environmental factors (e.g., more acidic field pH) and AMF species compatibility with the host plants. Therefore tests of the efficacy of mycorrhizal inoculation in the field are needed to confirm practical utility.

1.6. References

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1.6. Tables and Figures

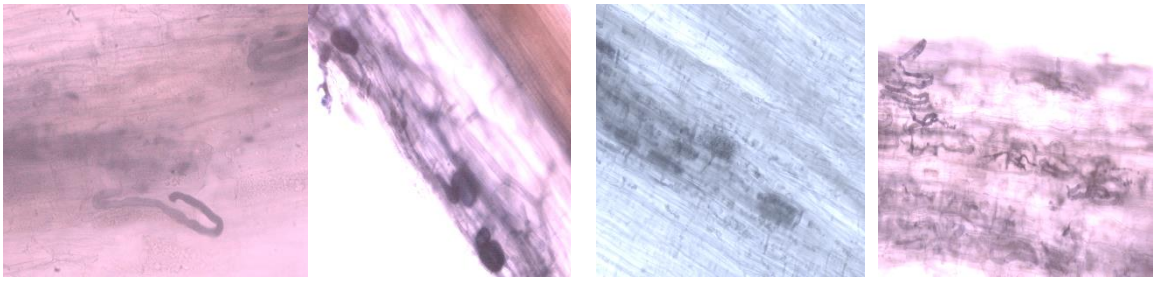
Table 1.1. % root length with AM structures (aseptate hyphae, vesicles, arbuscules) and non-AM fungal structures (septate hyphae) in the NWC stained roots with 200x magnification

Treatment	Aseptate hyphae	Septate hyphae	Vesicles	Arbuscules
Control	0± 0.0	1.9± 1.1	0± 0.0	0± 0.0
M[1]	9.9± 1.9	5.2± 2.3	0.8± 0.8	4± 1.9
F[1]	0± 0.0	1.3± 0.9	0.1± 0.1	1.2± 0.9
F+M	2.7± 1.1	15.3± 9.9	0.4± 0.4	2.4± 0.8
L[1]	0± 0.0	0± 0.0	0± 0.0	0± 0.0
L+M	11.7± 2.6	0± 0.0	0.8± 0.3	5.5± 1.9
L+F	0± 0.0	0.3± 0.3	0± 0.0	0± 0.0
L+F+M	2.7± 0.9	1.2± 0.7	0± 0.0	1.1± 0.7

[1] M: mycorrhizal inoculation; F: fertilization; L: liming

Table 1.2. P values for treatment effects on AM and non-AM fungal structures in the NWC stained roots. Abbreviations as in Table 1.1.

Treatment	Aseptate Hyphae	Septate Hyphae	Vesicles	Arbuscules
L	0.6708	**0.0373**	0.6037	0.7154
F	**<.0001**	0.3031	0.2552	0.1073
M	**<.0001**	**0.0893**	0.0514	**0.0002**
L*F	0.5520	0.4434	0.6037	0.1766
L*M	0.6708	0.1208	0.7553	0.6666
F*M	**<.0001**	0.2775	0.1795	**0.0176**
L*F*M	0.5520	0.3493	0.7553	0.5733



a) b) c) d)

Fig 1.1. The principles structures of AM fungi (a-c) and other root fungi (d), observed by clearing the roots tissues then staining roots with Chlorazol E. Black with 200X magnification: a) aseptate hyphae; b) vesicles; c) arbuscules; d) septate hyphae (non-AM fungi).

Table 1.3. P values of growth variables of the NWC seedlings. Abbreviations as in Table 1.1.

Treatments	Height (cm)	Diameter (cm)	Root Biomass (g)	Shoot Biomass (g)	Total Biomass (g)	Root Shoot Ratio
L	0.1508	0.6537	0.1807	**0.0100**	**0.0021**	0.2006
F	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
M	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0331**
L*F	**0.0309**	0.4070	**0.0072**	**0.0465**	**0.0178**	0.1451
L*M	0.1998	0.8217	**0.0034**	0.3693	**0.0005**	**0.0335**
F*M	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0431**
L*F*M	0.1334	0.8036	0.4054	0.6211	0.3414	0.2828

Table 1.4. P values of foliar nutrient variables of the NWC seedlings. Abbreviations as in Table 1.1.

Treatment	%N	%P	%Cu	N Content	P Content	Cu Content	N:P Ratio
L	0.0942	**0.0179**	0.0570	**0.0072**	**0.0263**	0.4620	0.6272
F	**0.0004**	**<.0001**	**0.0208**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
M	0.9393	**0.0017**	0.0552	**<.0001**	**<.0001**	**<.0001**	**0.0139**
L*F	0.3606	0.3818	0.2655	0.8996	0.5896	0.7241	0.0869
L*M	**0.0449**	**0.0337**	0.7406	0.3874	0.1655	0.4648	0.5446
F*M	**0.0172**	0.6661	**<.0001**	**<.0001**	**0.0025**	**0.0009**	**0.0145**
L*F*M	0.1702	0.4349	0.1025	0.3030	0.5844	0.9190	0.9490

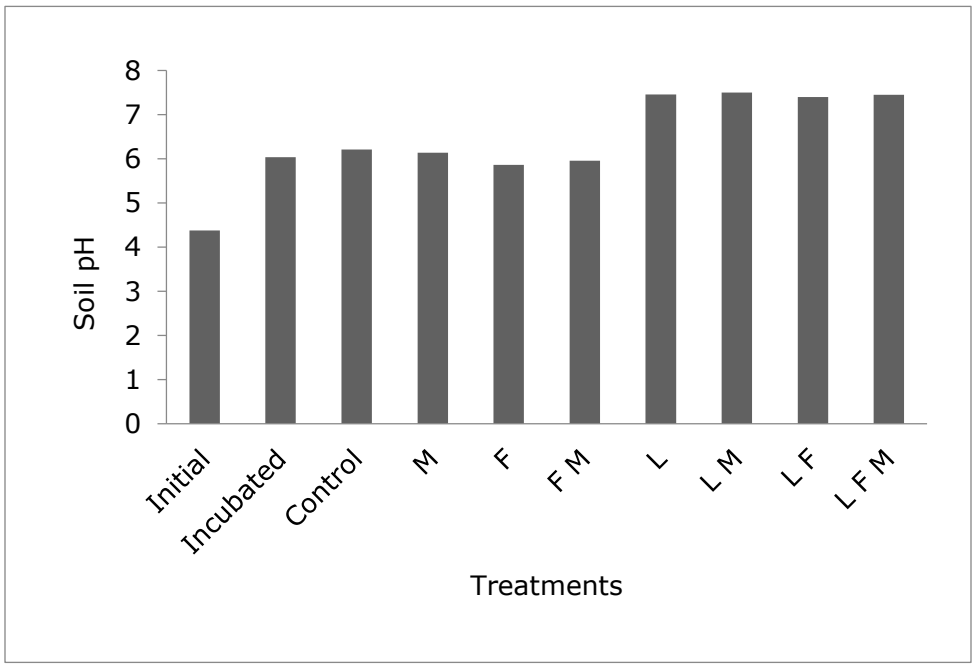


Fig 1.2. pH of pooled samples of the growth media used in the experiment(Control: no treatment. Initial = prior to experiment. Incubated = limed prior to the experiment. Others represent final pH of pooled soil samples. pH of peat increased over the course of the experiment. Abbreviations as in Table 1.1.

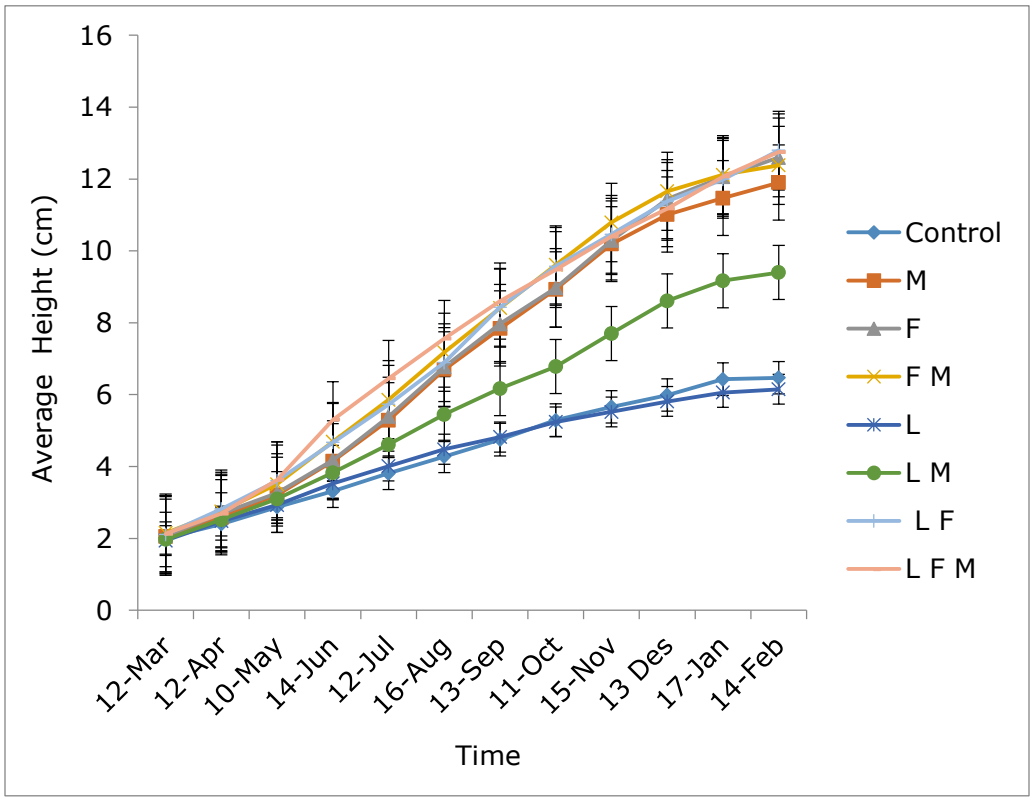


Fig. 1.3 Average height of the NWC seedlings within 11 months (Control: no treatment, M: mycorrhizal inoculation, F: fertilizer, FM: fertilization*mycorrhizal inoculation, L: liming, LM: liming*mycorrhizal inoculation,LF: liming*fertilization, FM:liming*fertilization*mycorrhizal inoculation). Error bars indicate standard error.

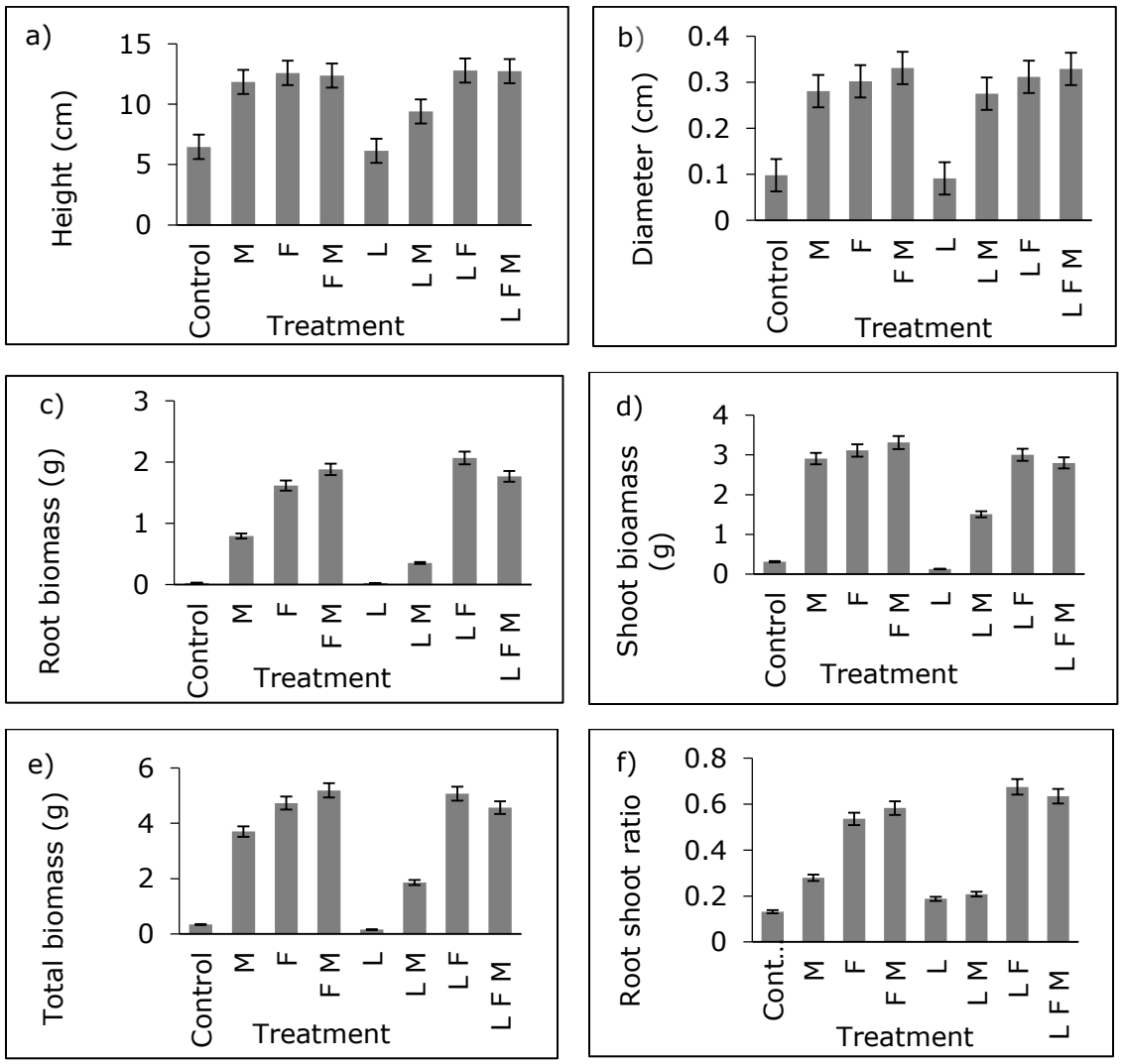


Fig 1.4. Effect of mycorrhizal inoculation, liming, and fertilization on growth of the NWC seedlings on: a) height; b) diameter; c) root biomass; d) shoot biomass; e) total biomass; f) root shoot ratio. See Table 1.3 for significance tests. Abbreviations as in Table 1.1. Error bars indicate standard error.

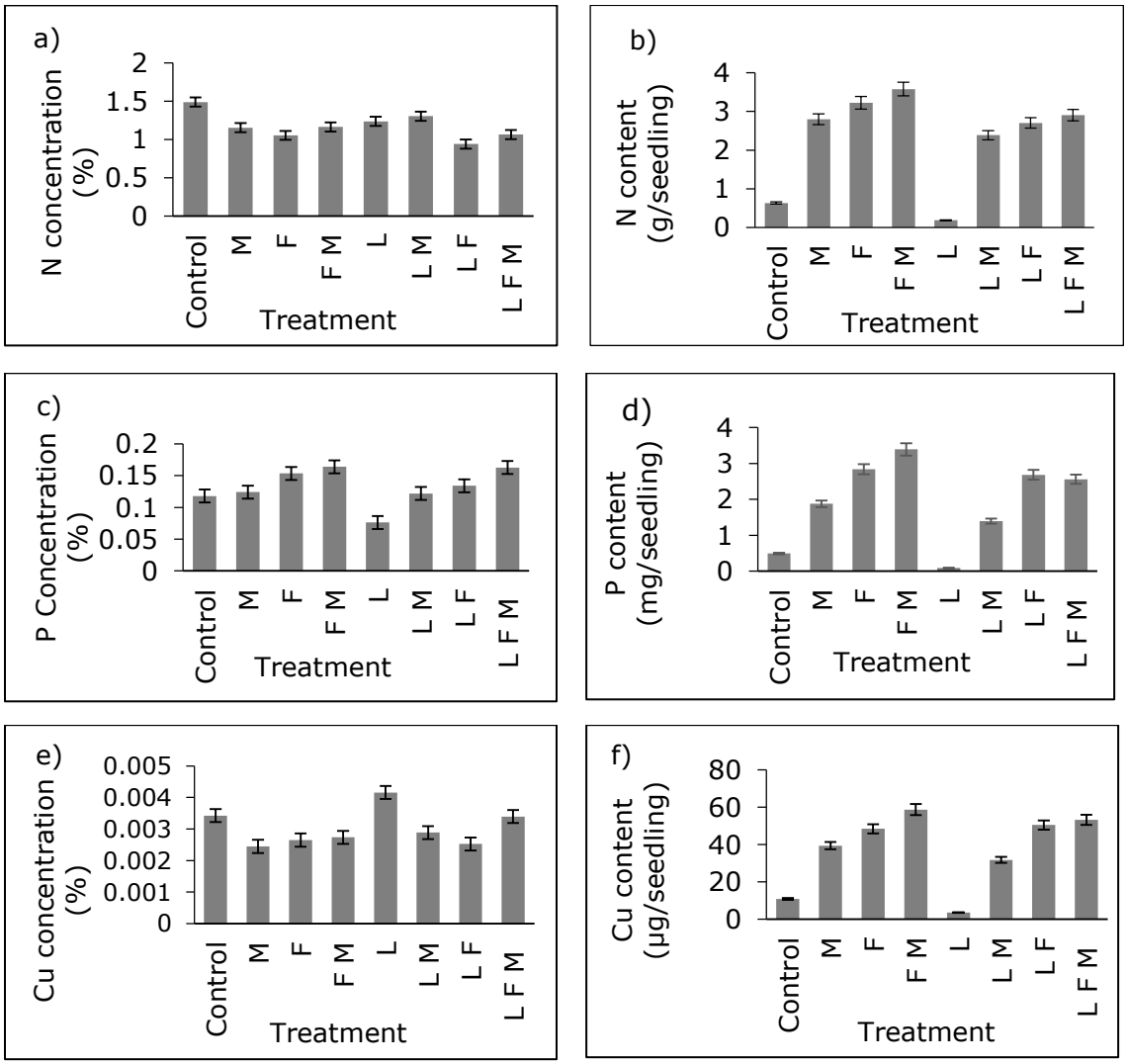


Fig 1.5. Average nutrient status of the NWC foliage: a) N concentration; b) N content; c) P concentration; d) P content; e) Cu concentration; f) Cu content. See Table 1.4 for significance tests

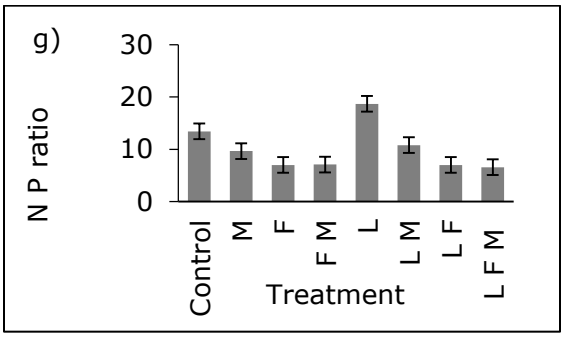


Fig 1.5 (cont'd). Average NP ratio of the NWC foliage. See Table 1.4 for significance tests.

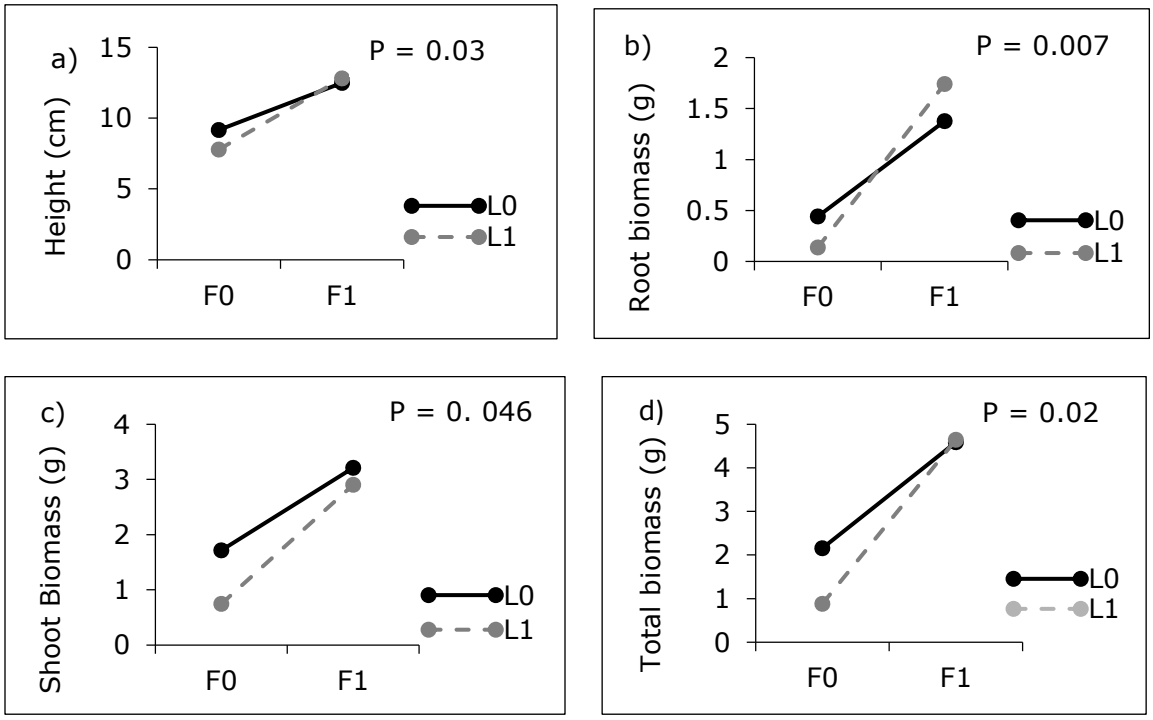


Fig 1.6. Interaction plots between fertilization and liming on height (a); root biomass (b); shoot biomass (c); and total biomass (d) (L0: unlimed, L1: limed, F0: unfertilized, F1: fertilized)

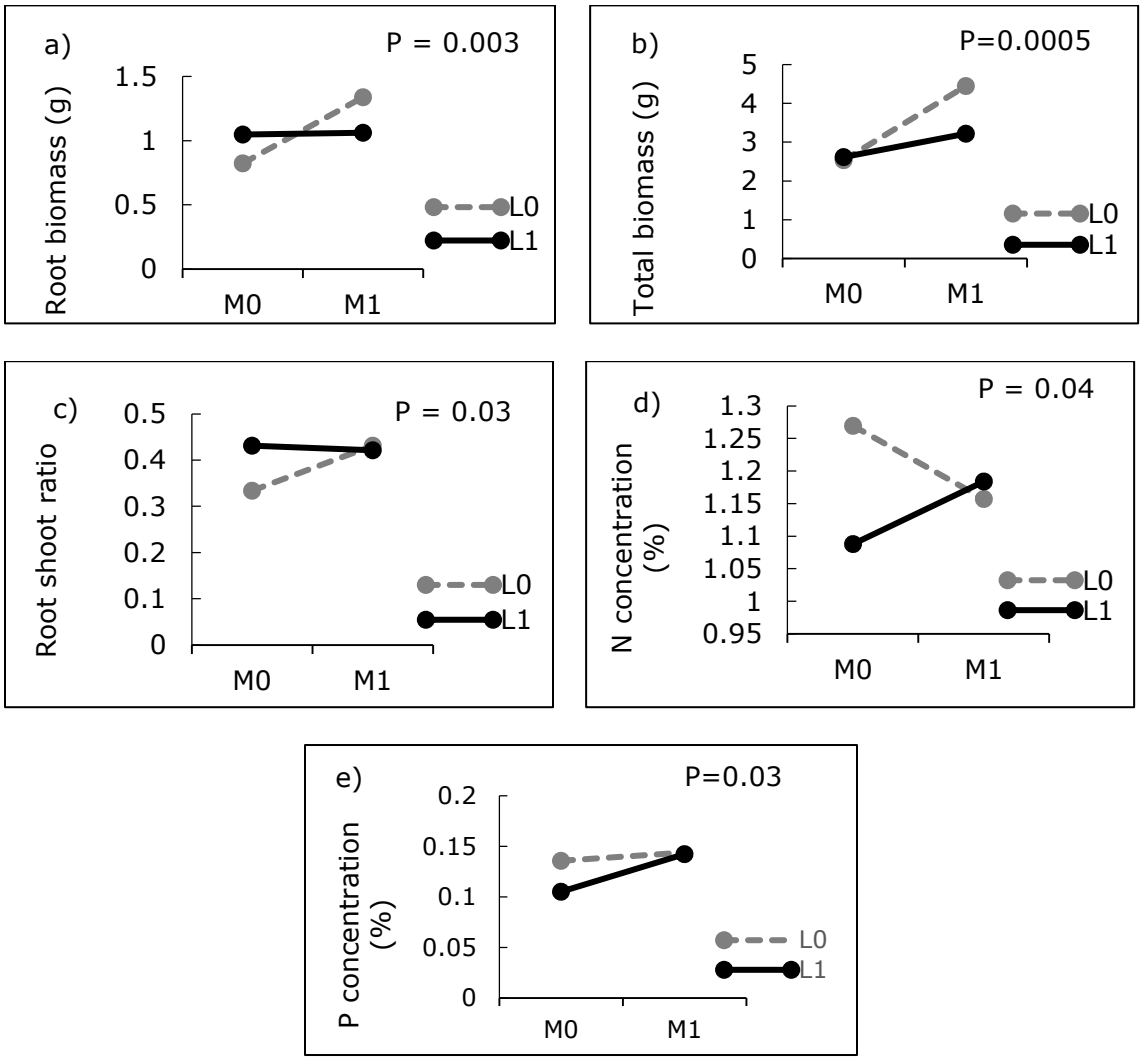


Fig 1.7. Interaction of liming with inoculation on : a) root biomass; b) total biomass; c) root shoot ratio; d) N concentration, and e) P concentration. (L0: unlimed, L1: limed, M0: uninoculated, M1: inoculated)

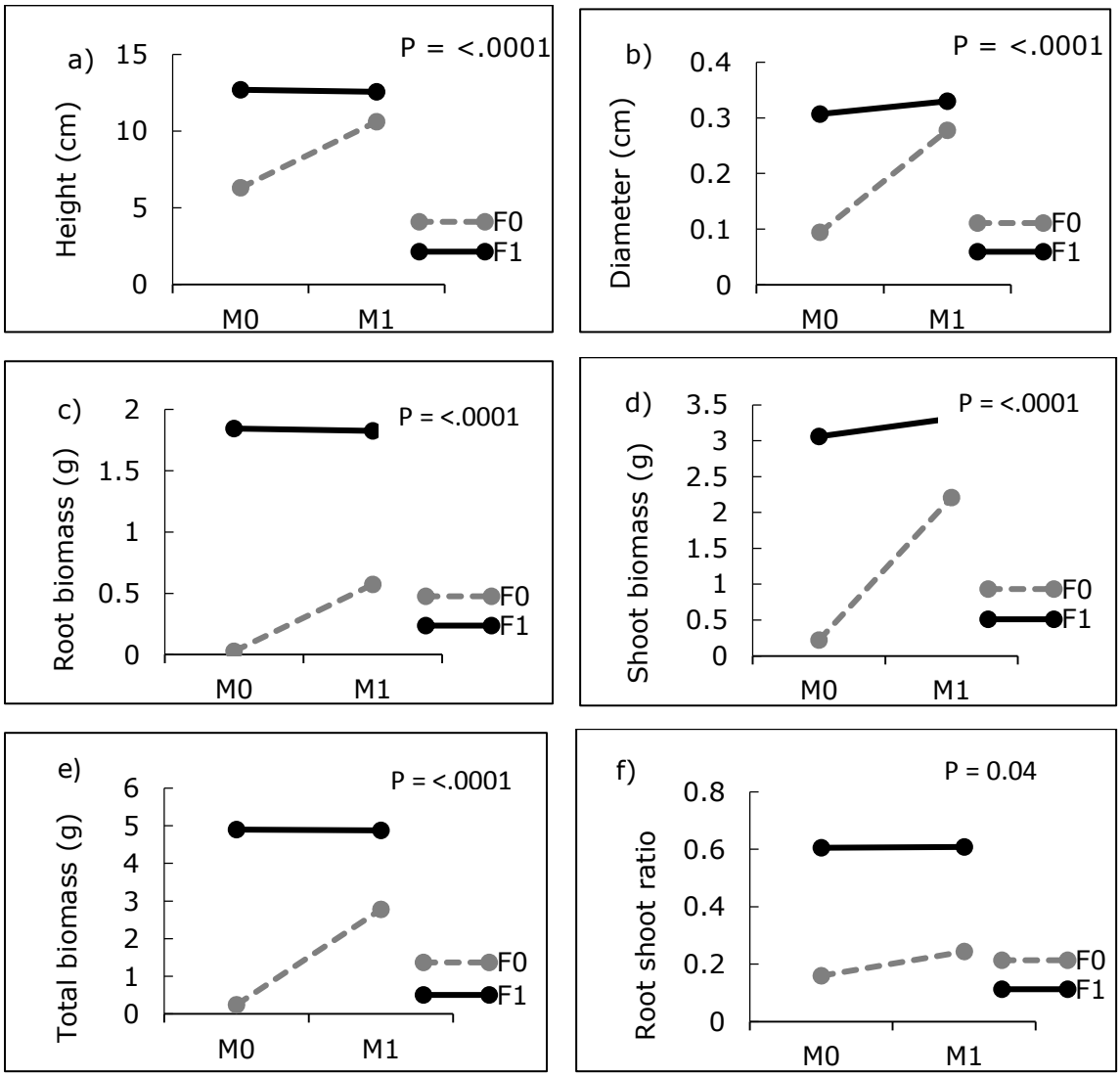


Fig 1.8. Interaction of fertilization with mycorrhizal inoculation on the seedling growth: a) height; b) diameter; c) root biomass; d) shoot biomass; e) total biomass; f) root shoot ratio. (M0: uninoculated, M1: inoculated, F0: unfertilized, F1: fertilized)

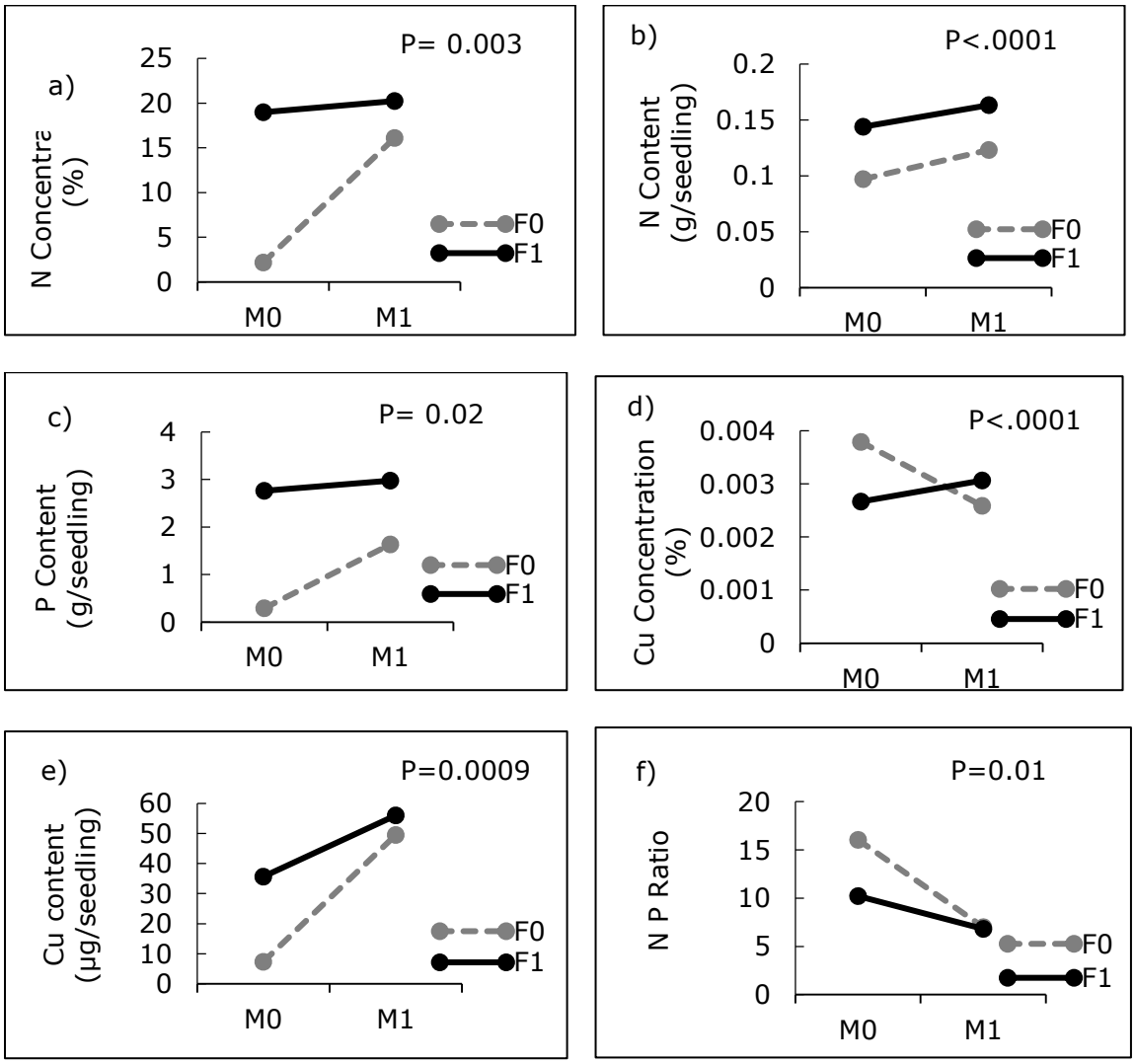


Fig 1.9. Interaction of fertilization with mycorrhizal inoculation on nutrient acquisition of the seedling foliar: a) N concentration; b) N content; c) P content; d) Cu concentration; d) Cu content, (k), and N P ratio (l). (M0: uninoculated, M1: inoculated, F0: unfertilized, F1: fertilized)

Chapter 2. The effect of arbuscular mycorrhizal inoculum, AM host proximity, and other environmental factors on growth and survival of *Thuja occidentalis* seedlings in a poor fen[2]

2.1. Abstract

Northern white-cedar (NWC) is occasionally found in acidic peatlands. As an arbuscular mycorrhizal (AM) plant, its establishment in such ecosystems could be mediated by limiting inoculum of AM fungi. We predicted that several factors play important roles to support NWC survival and growth, such as native AM inoculum, AM plant proximity, and several environmental factors (light, pH, water table depth, peat bulk density). We conducted a field study to examine the effect of these factors on the survival and growth of NWC seedlings. Our findings indicated that AM inoculant had no significant effect on survival. Light was the only significant predictor of survival, with higher light associated with greater seedling survival. Inoculation treatment had no significant effect on seedling growth and nutrient concentrations, either in single treatment or in interaction with other abiotic factors except with water table depth on relative growth rate. However, seedlings closer to AM trees showed higher growth and increased foliar nutrient concentration. Reduction of water table depth and higher pH were associated with greater plant growth and nutrient concentration. Higher light correlated with greater plant growth but reduced nutrient concentrations. The best model predicting plant growth and nutrient concentration involved light intensity, metrics of AM plant proximity (distance to hosts, PCA of plant community), soil pH, and water table depth. We conclude that using AM

[2] The material contained in this chapter is in preparation for submission to a journal.

inoculant did not improve survival rate, growth or nutrition of NWC seedlings, yet proximity to living AM host plants was associated with improved growth and nutrition. Further analysis is needed to determine whether the latter was due to mycorrhizal or other environmental factors. It may be important to consider proximity to AM hosts in peatland restoration projects.

2.2. Introduction

Northern white-cedar (NWC; *Thuja occidentalis* L.) is an important tree species in the northeastern United States and eastern Canada due to its various economic, social, spiritual, and ecological values (Johnston, 1990, Boulfroy et al., 2012). It occurs commonly as pure stands or mixtures in uplands (mesic mineral soils) and poorly drained lowlands (organic soils) (Johnston, 1990; Hannah, 2004; Hofmeyer et al., 2009, Larouche et al., 2011; Boulfroy et al., 2012; Man et al., 2013). In lowland peatlands, this species is generally found in association with black spruce (*Picea mariana*), balsam fir (*Abies balsamea*), and tamarack (*Larix laricina*) (Hofmeyer et al., 2009; Man et al., 2013).

Despite the fact that NWC is commonly found in high pH, nutrient rich peatlands, often called peat swamps (Hannah, 2004), it can sometimes be found in oligotrophic peatlands (forested poor fens) with organic soil, low pH and low nutrient availability. The nutrient impoverishment of poor fens limits the vascular plant species able to grow and survive, such as dwarf shrubs in the *Ericaceae,* some small insectivorous species, and few trees in the *Pinaceae* (Thornmann et al., 1999; Nordbakken et al., 2003; Thormann, 2006).

NWC is symbiotic with arbuscular mycorrhizal (AM) fungi (Brundrett et al., 1989, Matthes-Sears et al., 1992; Bainard et al., 2011; this dissertation Chapter 1).

AM fungi play an important role in nutrient poor soils (Smith et al., 2011). Generally, vascular plants hosting ericoid mycorrhizal fungi (Ericaceae) and ectomycorrhizal fungi (Pinaceae) are prevalent in acidic peatlands, whereas arbuscular mycorrhizal hosts are not abundant in northern acid peatlands (Thormann, 2006). AM plants are commonly found in mild climates with phosphorus poor soils in the northern hemisphere, in contrast to ectomycorrhizal that predominantly occurs in colder climates with low nitrogen soils (Allen et al., 1995; Smith and Read, 2008). Both ericoid and ectomycorrhizal fungi are thought to be especially good at mobilizing organic nitrogen, which can be critically important in these often N-limited peatlands. Extracellular enzymes produced by ericoid and selected ectomycorrhizal fungi promote their abilities to degrade and decompose proteins and chitins to provide nitrogen (Read et al., 2003). Meanwhile, AM fungi have conventionally been recognized to be exclusively able to mobilize inorganic nutrients (Smith and Read, 2008). However, recent studies reveal that AMF were able to take up organic nitrogen from organic sources (Nasholm et al., 1998; Whiteside et al., 2009; Hodge and Fitter, 2010; Talbot & Treseder, 2010) and some species can use several organic nitrogen derivates (amino acids) (Hawkins et al., 2000; Cappellazzo et al., 2007). Whiteside et al. (2010) confirmed that AMF gained organic nitrogen in recalcitrant and labile forms.

AM fungi are very important in the earliest stage of AM plant life cycles, especially in nutrient impoverished sites and disturbed ecosystems. Absence of propagules of AM fungi potentially reduces nutrient supply for the plants in such environments. Lack of AMF inoculum triggers reduction of AM plant survival and growth, particularly where ectomycorrhizal and ericoid plants dominate (Weber et al., 2005).

Environmental factors that influence plant growth in peatlands might also affect AM effectiveness. Soil pH, fertility, water table depth, bulk density, and light intensity might have effects on mycorrhizal functions where AM fungi increases their benefits under limited soil nutrients and reduce their benefit under limited light (Johnson, 2010). For instance, root colonization by AM fungi declined under shading, and abundance of hypha in soil decreased with fertilizer addition (Shi et al., 2014). Likewise, increasing water availability reduced AM colonization (Miller, 2000; Escudero and Mendoza, 2005).

Understanding the required conditions for AM colonization and the factors affecting the AM fungi functions in the earliest stage of NWC establishment is crucial for peatland restoration with NWC. Currently, NWC is undergoing restoration trials in several states to try and reestablish populations (e.g., Kangas et al., 2015). To determine the importance of AM fungi in colonizing NWC seedlings, we examined several factors that potentially affect NWC growth and survival including AMF inoculant and environmental factors. The objectives of our study were: 1) to determine effect of native AM inoculant, 2) to test effect of measures of AM plant proximity (distance to, basal area of, and % cover of AM host species), and 3) to determine effect of environmental factors (soil pH, water table depth, bulk density, and light intensity).

We hypothesized that; 1) native AM inoculant would increase growth and survival of NWC seedlings, 2) higher AM host plant abundance would positively affect growth, nutrition, and survival of NWC seedlings, and 3) Light, water table depth, soil pH, and cover of Ericaceae would affect growth and survival of the NWC seedlings.

2.3. Materials and Methods

2.3.1. Study site

The study took place in a peatland near Painesdale, Houghton County, MI (N 47.01349°, W 88.43082°). Cumulative precipitation during the study period (from 1 September 2013 to 1 November 2014) was 679 mm (NOAA, 2015). The site is a partially forested poor fen with hummock and hollow microtopography, covered mainly by dwarf shrubs in the Ericaceae and *Sphagnum* mosses. The peatland margin was dominated by NWC, tamarack *(Larix laricina* (Du Roi) K. Koch) and tag alder (*Alnus* spp), and NWC and other trees extended out partway into the peatland from one margin. Soil pH range of the site ranged between 3.7 and 5.0 (Appendix Table 2.1).

2.3.2. Experimental treatment

Our goal was to set up plots over the range of conditions in the peatland, including areas with and without NWC and other AM hosts present (Appendix Table 2.1). We established a 200 m-long transect set perpendicular from to the edge of the peatland toward its center. This transect extended from the forested margin of the peatland to the open fen in the center dominated by *Sphagnum* and Ericaceae. At 10, 50, 100, 150, and 200 m along this transect we established several 100 m-long secondary perpendicular transects, with exception the 10 m transect, which was only 70 m-long because of the shape of the peatland. Over the length of these secondary transects we randomly placed replicate plots within each 5 m interval. Each plot had four points in a crossed design with the length of each axis 2 m. At each point we dug a 15x15x20 cm hole to plant each NWC seedling. In total, there were 396 NWC

seedlings (99 plots x 4 seedlings per plot). One year old NWC seedlings were obtained from the J.W. Toumey Nursery, MI, and maintained until time of planting in the greenhouse at the School of Forest Resources and Environmental Science for 6 months prior to planting. The seedlings were watered daily using tap water and temperature setting was 22°-24°C. The greenhouse conditions were 16 hours day length using supplemental lighting via Halco metal halide lamps (Prolume MP 400/BU.

We planted the seedlings on October 29, 2013. At planting seedlings had an average height and diameter of 26.7 cm (SE = 0.28cm) and 3.5 mm (SE= 0.1mm), respectively. Two of the seedlings in each plot were treated with native mycorrhizal inoculum and two were uninoculated. Native mycorrhizal inoculum was obtained by collecting NWC fine roots from rhizospheres of NWC trees in the site. Fresh fine roots (20 g) added to the planting hole at time of planting was used as inoculum for each NWC seedling, and as a control, 20 g of pasteurized fine roots were added into the holes of the uninoculated seedlings. The fine roots were pasteurized in an oven at 80° C for 30 minutes. The seedlings were grown in the field for ~12 months.

2.3.3. Data collection

Pre-harvest.

Height and diameter were measured three times: at planting, in June (~8 months after planting), and at the end of the experiment (~12 months after planting). Seedling survival was measured at the end of the experiment.

To estimate percent of full sunlight reaching each seedling, we measured photosynthetically active radiation using an Apogee Quantum Flux MQ-200 PAR Meter at five points at the top of the canopy of each seedling, and simultaneously

measured full sunlight at an open location using an AccuPAR model LP-80 Ceptometer. Water table depth was measured using a perforated PVC pipe inserted into the hole at the center of each plot. Percent cover classes of mosses, herbaceous plants, dwarf shrubs and seedlings were measured using a PVC quadrat frame (1 m^2) centered on the seedling. Percent cover was grouped into 6 classes (1= <1%, 2=1-5%, 3=5-25%, 4=25-50%, 5=50-75%, 6=>75%) based on ocular estimate of the percentage of coverage of the species in the frame.

Post-harvest

In September of 2014 half of the seedlings (one inoculated and one uninoculated; 198 total) from each sampling plot were harvested. A subsample of the fine roots (0.3 g) from each seedling sample were picked and weighed fresh and used to estimate mycorrhizal colonization. Root, stem and leaf biomass were determined separately after oven drying at 65^{o} C to a constant weight.

Soils from each seedling planting location were sampled by coring at three points around the seedling planting holes to 20 cm depth using a 4 cm diameter steel corer. Bulk density was estimated with drying soils and calculating mass per unit volume of soil samples. Soil pH was measured with a mass ratio of 1(dry peat):40 (DI H_2O) using a pH meter (Denver Instrument Model 220, Denver Instrument, Arvada, CO, USA).

Dried foliage was ground to a fine powder, and foliar nutrients consisted of N, C, P, and Ca were measured at the Soil Laboratory of SFRES, Michigan Tech. N and C were analyzed on Costech 4010 Elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) that calibrated with atropine. P and Ca analysis were

performed using a Perkin Elmer Optima 7000DV ICP-OES (PerkinElmer Inc., Waltham, MA, USA).

2.3.4. Data analysis

Survival (class response variable) by inoculation (class treatment) was analyzed using ChiSquare test in JMP Statistical Discovery (Version 12, SAS, Campus Drive, Cary, NC, USA). Survival vs. continuous independent variables was tested using logistic regression. Continuous dependent variables (growth and nutrient concentration) vs. continuous independent variables (light, pH, bulk density, water table depth, distance to AM hosts, and cover/basal area of mycorrhizal sources) were analyzed using regression methods (simple and all possible subsets multiple regression), and PCA (Principle Component Analysis) was run analyzed using JMP Statistical Discovery Version 12 (SAS Campus Drive Cary, NC, USA). PCA scores were rotated using Factor Analysis to align with primary axes, resulting in an output of factor scores that could be used as predictors in multiple regression. We ran a species-level PCA only to describe the community-level patterns, and one based on cover and basal area of different mycorrhizal types to use in regressions predicting NWC success.

2.4. Results

2.4.1. PCA of plant cover

To reduce the dimensionality of ground cover and tree community data as a predictor of seedling performance, a PCA was performed (Fig 2.1 and 2.2). Ground cover of AM hosts *T. occidentalis, Symphoricarpos albus*, *Drosera spp.*, and *Nemopanthus* were clustered and had positive scores along the Factor 1 axis that accounted 14.8 % of total variance (Fig 2.1). In a second PCA on mycorrhizal and

other cover classes and basal area of different mycorrhizal trees, measures of AM cover and basal area had positive scores on Factor 1, whereas ERM cover had negative scores (Fig. 2.2, Table 2.2). This axis accounted 33.4% of total variance (Fig 2). PCA factor 1 was used as a predictor of seedling performance in the regression models.

2.4.2. Survival rate

Inoculated seedlings had slightly higher survival rate than uninoculated seedlings, but this effect was not significant ($P = 0.405$) where 154 inoculated seedlings (76%) and 147 uninoculated seedlings (72%) survived through the end of the observation. In logistic regression analysis, light was the only significant predictor of survival ($P < 0.001$; Table 2.3), with greater amount of light correlated with greater seedling survival (Fig 2.3).

2.4.3. Seedling growth and nutrient acquisition

Paired t-tests and Wilcoxon Signed Rank Tests showed no significant effect of inoculation on any of the seedling responses (Table 2.4). Stepwise analysis resulted in no significant effect of inoculation alone and the interaction of inoculation with the proximity AMF tree, environmental factors and plant community, except for a weak interaction with water table depth (WTD) on the relative growth rate (RGR) (Table 2.7). Meanwhile, distance to the nearest AMF tree (logdistAM+1), plant community factor 1 scores, and all abiotic environmental factors except bulk density (BD), showed significant relationship on the response variables when they were analyzed with inoculation treatment (Tables 2.5 - 2.10). Seedlings closer to AM trees had higher growth and nutrient concentrations (Table 2.5, Fig 2.4). Soil pH had a positive

relationship with seedling growth increment, foliar %N, %P, and N:P ratio (Fig 2.5). Increasing water table depth had a negative relationship with N & P concentrations (Table 2.7, Fig 2.6). Increasing light was a predictor of increased growth but reduced N, P, and Ca concentrations (Table 2.9, Fig 2.8). Factor 1 of the PCA of plant communities (positively related to AM host cover and negatively to Ericaceae cover) was associated with greater plant nutrient concentrations (Table 2.10, Fig 2.9).

Using multiple regression, the best models (lowest AICc value) predicting growth and nutrient acquisition most commonly included light intensity, metrics of AM plant proximity (distance to the closest AM tree and Factor 1 of the PCA of plant communities), soil pH, and to a lesser extent depth to water table (Table 2.12, Fig 2.10).

2.4.4. Relationship between AM plant proximity and other predictors

AM plant proximity had significant relationship with light intensity and ERM cover (P=0.0002), whereas no significant relationship with soil pH, water table depth, and bulk density (Table 2.11).

2.5. Discussion

2.5.1. AM Fungi: inoculation success and effectiveness

Our AM inoculation results lead to the rejection of the hypothesis that native AM inoculant increased growth and survival of the NWC seedlings. There are two possible reasons for this. First, the inoculation might have been unsuccessful. Second, the inoculation might have been successful but the mycorrhizas might have been ineffective under the environmental conditions encountered.

Regarding the first possibility, there may have been no AM colonization. Results on the success rate using native inoculum vary. While many studies reported that native AM fungi are more effective than non-native in plant colonization due to their adaptation to the plant site condition (Caravaca et al., 2003; Oliveira et al., 2005; Querejeta et al., 2006), other studies found the opposite (Trent et al., 1993; Calvente et al., 2004). Schreiner (2007) suggested that the relative effectiveness of native vs. non-native AM inoculant is still poorly understood. Moreover, Afek et al. (1990) and Werner & Kiers (2014) suggested that AM colonization in the field might be less successful than under greenhouse condition due to AMF density and environmental factors.

Regarding the second possibility, that the AM fungi were ineffective under the study conditions, Fitter (1985) found that AM fungi field studies showed considerable divergence in the effectiveness results. Since AM fungi effectiveness may vary depending on the plant and fungal condition, it is possible that plant, AM fungi, or environmental factors could contribute to the non-significant results. Given that we used inoculum from NWC it is unlikely that there was a host-fungal incompatibility. However, it is possible that under the nutrient stresses of acidity and low nutrient availability in the Ericaceae-dominated areas of the peatland the AM fungi were unable to provide significant benefit to the NWC seedlings. If that is the case, then AM inoculum might be ineffective in invasion of acid peatlands. Our greenhouse experiment (Chapter 1) indicated that AM fungi were effective under somewhat acidic conditions. However, although the initial pH was quite acidic (4.4), pH increases caused by watering with tap water led to a final pH of around 6, so it is unclear whether benefit would have accrued under more acidic conditions. Furthermore, the greenhouse experiment took place in the absence of Ericaceae or

other plant competitors. Further manipulative experiments teasing apart pH and competitor impacts on seedling success would be informative.

In addition to the above factors that could influence the experimental outcome, different size of seedlings (height, diameter, root condition) and length of time of the seedlings in the nursery prior to inoculation might also determine AM fungi effectiveness. We used un-inoculated seedlings that originated from the nursery with various sizes. The seedlings were regularly fertilized in the nursery, although not in the 6 months prior to outplanting. John (1996) advocated that greatest benefits of AM inoculation will be found in the earliest stage of the plant development. Werner & Kiers (2014) found that it is likely host plants have space limitation in their roots where AM fungi are not able to invade the roots due to occupation from the previous colonizer. Meanwhile, Cano & Bago (2005) and Bennett & Bever (2009) found that there was profound competition across AM fungi for root space.

AM fungi have are associated with stressful environment and their efficacy is strongly affected by environmental factors (Smith and Read, 2008). Our finding showed there was no significant effect between AM inoculation and other environmental factors except perhaps on water table depth. However, this result was so weak, especially in the context of multiple tests, that we are cautious in its interpretation, even though based on the correlation graphs showed AM inoculated plants performed greater than un-inoculated plants.

2.5.2. AM host proximity and plant community effects

In contrast with our result on AM inoculation, we found that proximity to AM host plants was positively associated with growth and nutrient acquisition. The

distance effect was non-linear, with much greater effects within 10m of AM host plants. There are two likely alternative explanations for this pattern. First, it is possible that the NWC seedlings benefited from access to the common mycorrhizal network and high inoculum density near other AM host species. Alternatively, it is also possible that the environmental conditions near AM hosts (pH, nutrients, light, others unmeasured factors) are more favorable to NWC compared to conditions farther away.

Regarding the first alternative, it is possible AM fungi on AM hosts near the NWC seedlings infected and colonized their roots, leading to a positive AMF-mediated interaction by proximity to AMF host plants. Jastrow and Miller (1993) suggested that presence of neighbor-plants led to mycorrhizal network formation between the plants. Dickie et al. (2005) reported that seedlings showed the best performance within 15.7 m of host trees of the same mycorrhizal type, where high mycorrhizal infection and high nitrogen uptake occurred. Ronsheim & Anderson (2001) stated that association with neighbor AMF plants benefit. Lyford (1980) suggested that increasing mycorrhizal infection occurred in the root zones of AMF trees.

Much of our study peatland was dominated by ericoid mycorrhizal and ectomycorrhizal plants which do not share mycorrhizal fungi with AM plants. Presence of other AM plants could therefore play a pivotal role on seedling growth. Our finding showed other AM plants were positively associated with NWC growth and nutrient acquisition. It is possible the NWC seedlings roots were colonized by hyphae or fungal propagules of other AM plants located near the NWC seedlings. On the other hand, AM hyphae of the NWC seedlings were not able to connect with mycorrhizal network of other mycorrhizal types (ErMF and ECMF). Molina et al. (1992) suggested that compatibility within a single host species commonly occurs

with a specific mycorrhizal type, and this appears to be true for NWC (Brundrett et al., 1989; Sears et al., 1992; Bainard et al., 2011). Limited number of NWC trees in the study site may be caused by insufficient dispersal of AM fungi where their availability was restricted by presence of the appropriate hosts. This interpretation is only likely if our inoculations were successful, or if subsidy of the mycorrhizal network via common mycorrhizal networks is necessary for benefit. Similar condition occurred in red cedar seedlings where deficiency of dispersal AM trees among ectomycorrhizal plants may have restricted red cedar establishment (Weber et al., 2005).

Regarding the second alternative, we found there was a significant relationship between AM plant proximity with light and ERM (ericoid mycorrhizal) plant cover (R^2=0.1982; P =0.0002 for each). Increasing distance from the nearest AM plant was associated with greater light and greater ERM cover. However, it seems unlikely that the light environment closer to the AM trees was favorable for seedling growth, because of our previous finding that seedlings grew faster in higher light. Meanwhile, ERM cover was lower near AM hosts. This condition could have high benefit for the NWC seedlings since less ERM cover potentially reduced competition for limited nutrients, particularly N. We also found that increasing ERM cover was associated with lower nitrogen concentration in NWC seedlings (R^2 = 0.0284, P = 0.0187). Therefore, it is possible that AM plants were able to maximize their functions in mobilizing nutrients for the NWC seedling with less competition with ERM plants. Hence, we cannot rule out possibility that the increase growth of the NWC seedlings near AM plant might be associated with ERM cover. Of course, these two alternative explanations (greater benefit from common mycorrhizal networks and less competition) are not mutually exclusive.

2.5.3. Other environmental effects

Our finding showed light intensity had a positive relationship with plant growth. Light intensity will increase photosynthetic rates and supply more carbon to the roots. Increasing shading in the nearest AM trees potentially reduced plant growth and nutrient acquisition. Weber et al. (2005) reported increasing growth of red cedar seedlings under high light treatments. High light is very important for seedling development and establishment, where their shoots and roots will be greater under full light in wet sites. It is likely that capillarity brought sufficient moisture to the roots of all seedlings in the present study. Meanwhile, reduction of nutrient availability under high light occurred possibly because there was nutrient pool dilution via greater growth.

Soil pH contributed positively to reducing N:P ratio. Increasing pH from 3.8 to 5.0 led to decrease N:P ratio that indicated increasing P availability. Johnston (1990) stated that NWC commonly occur on soils with pH 5.5-7.2. Bolan et al. (2003) reported that soil pH <4 potentially impair plants and soil microorganisms where it may stimulate toxic elements. Sumner et al. (1991) stated that some essential nutrients such as P, Mo, Ca, and Mg become less available in low pH soils.

We conclude that occurrence of AM fungi might be a crucial factor in peatland restoration especially in plant succession with NWC. Presence of AM plants may play an important role in seedling establishment, and so might determine success of seedling planting programs in peatland restoration projects.

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2.7. Tables and Figures

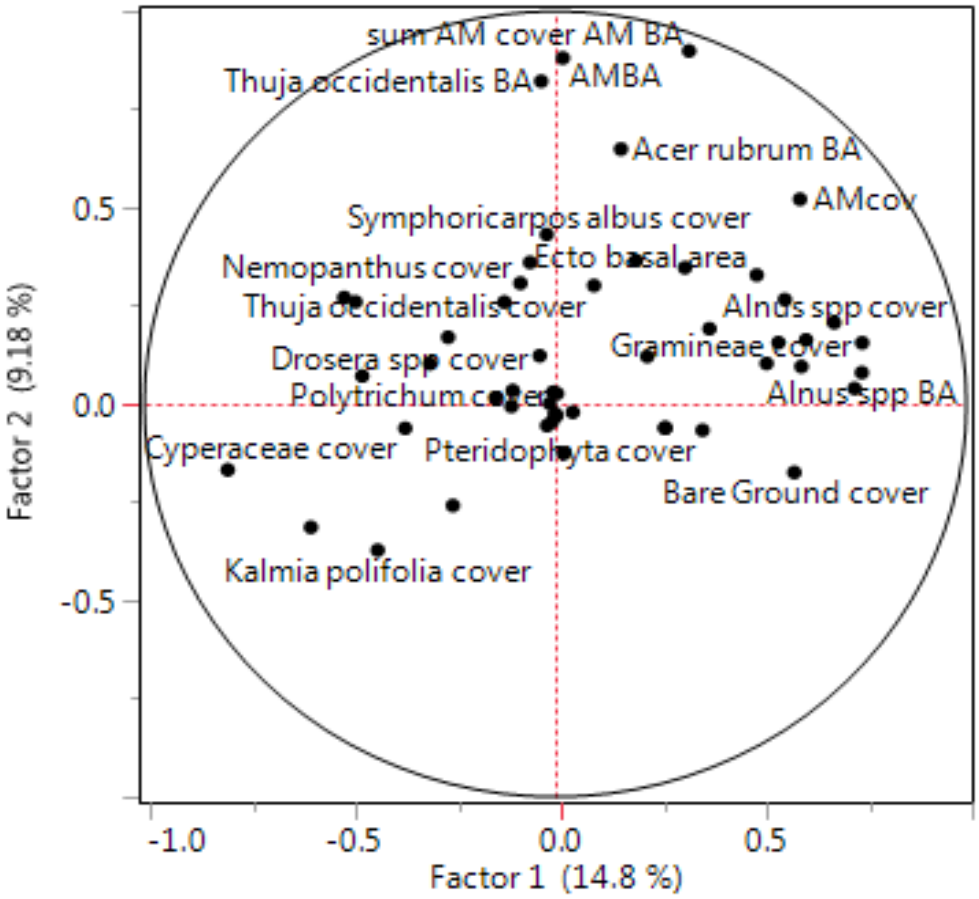

Fig 2.1. PCA of ground cover and basal area of plant community. Symols represent cover classes or basal area of species.

Table 2.1. Cover class loadings on the first two axes of the rotated species-level factor analysis of the plant community. BA = basal area

Species	Factor 1	Factor 2
Acer rubrum cover	0.196	0.365
*Alnus s*pp cover	0.678	0.207
Amelanchier cover	0.042	-0.020
Andromeda polifolia cover	-0.249	-0.258
Bare Ground cover	0.581	-0.173
Betula papyrifera cover	-0.013	0.001
Betula allegheniansis cover	0.266	-0.060
Bryophyta cover	0.598	0.095
Chamaedaphne calyculata cover	-0.594	-0.313
Cyperaceae cover	-0.365	-0.061
Cypripedium acaule cover	-0.145	0.016
Decomposed CWD cover	-0.001	-0.023
Drosera spp cover	-0.038	0.123
Gramineae cover	0.746	0.156
Kalmia polifolia cover	-0.432	-0.371
Larix laricina cover	-0.106	-0.005
Ledum groenlandicum cover	-0.261	0.170
Lichen cover	0.020	-0.124
Litterfall cover	0.558	0.265
Nemopanthus cover	-0.085	0.307
Open Water cover	0.513	0.103
Orchideceae cover	-0.005	0.030
Picea mariana cover	-0.020	-0.054
Polytrichum cover	0.004	0.0271
Pteridophyta cover	0.357	-0.066
Quercus rubra cover	0.266	-0.060
Sphagnum cover	-0.514	0.270
Symphoricarpos albus cover	-0.021	0.431
Thuja occidentalis cover	0.094	0.301
Typha latifolia cover	0.542	0.156
Undecomposed CWD cover	-0.008	-0.045
Vaccinium oxycoccos cover	-0.470	0.071
Vaccinium uliginosum cover	-0.124	0.259
AMcov	0.594	0.521
ECM cover	0.609	0.163
ERM cover	-0.797	-0.167
OM cover	-0.104	0.034
NM cover	-0.486	0.260
lichen cover	0.020	-0.124

Table 2.1. (cont'd)

Acer rubrum BA	0.159	0.649
Alnus spp BA	0.745	0.080
Betula papyrifera BA	0.223	0.121
Betula allegheniansis BA	0.374	0.191
Larix laricina BA	-0.305	0.103
Picea mariana BA	-0.061	0.360
Pinus strobus BA	0.002	-0.029
Thuja occidentalis BA	-0.034	0.821
Tsuga canadiensis BA	0.315	0.347
AMBA	0.018	0.880
Ecto basal area	0.489	0.328
sum AM cover AM BA	0.3252	0.898

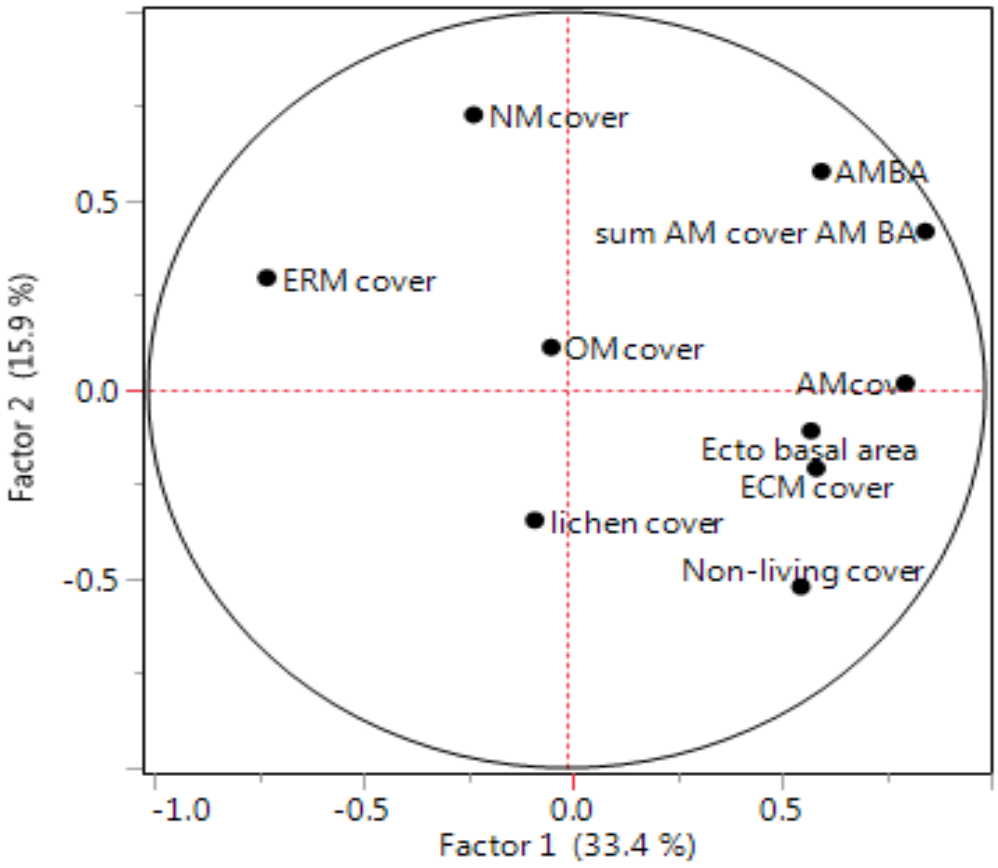

Fig 2.2. PCA of cover and basal area classes

Table 2.2. The loading of mycorrhizal type and other cover and basal area classes on the two axes of the rotated factor analysis.

Cover type	Factor 1	Factor 2
Arbuscular Mycorrhizal cover	0.809264	0.018045
Ectomycorrhizal cover	0.595983	-0.207510
Ericoid mycorrhizal cover	-0.716490	0.296521
Orchid mycorrhizal cover	-0.037057	0.112754
Non-mycorrhizal cover	-0.222074	0.727849
lichen cover	-0.076313	-0.344531
Non-living cover	0.559586	-0.520112
Arbuscular mycorrhizal BA*	0.608565	0.578145
Ectomycorrhizal basal area	0.583554	-0.108014
Sum AM cover AM BA	0.856789	0.419627

* BA = basal area

Table 2.3. Effect of inoculation and abiotic factors on survival of the seedlings

Variables	ChiSquare	P Value
Inoculation	0.6935	0.405
Soil pH	3.6766	0.055
Water Table Level (WTL)	0.6256	0.429
Bulk Density (BD)	0.1712	0.679
% Light	10.695	**0.001**
AM Plant Index	0.6314	0.429
AM Plant Proximity	2.5357	0.111
ERM cover	2.5501	0.110

P value <0.05: null hypothesis rejected, chi square < P value: null hypothesis accepted

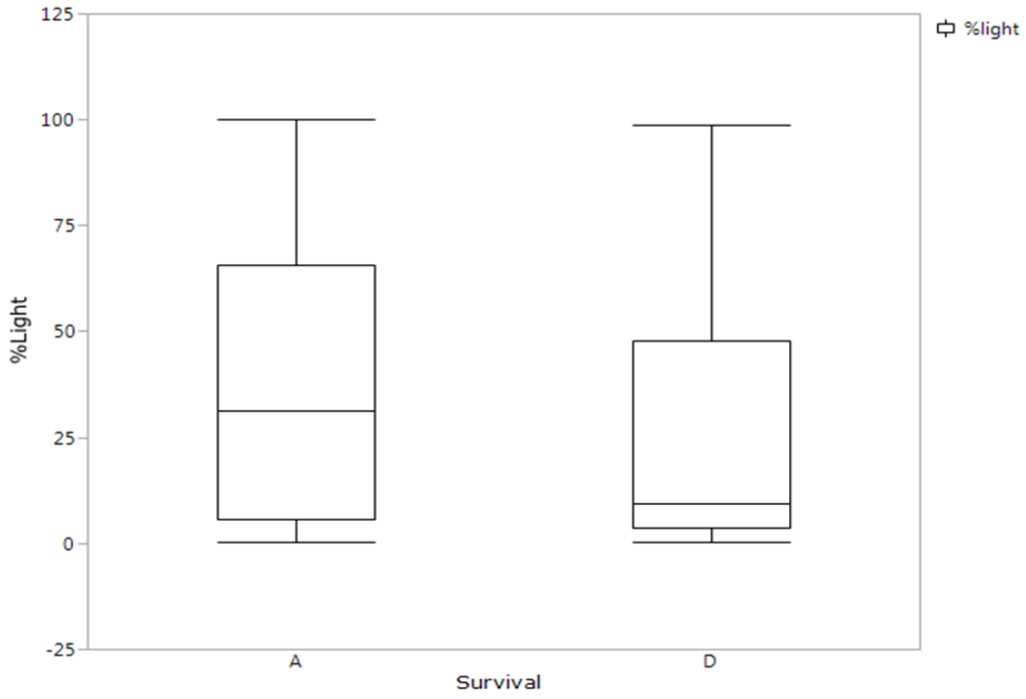

Fig 2.3. One way analysis of light intensity (% full sunlight) by survival (A=alive, D= dead). Boxes represent 25% quantiles, bars represent range.

Table 2.4. Summary of P value of effect of inoculation on the seedling response variables. T tests were used unless the data did not meet the assumptions of the test, in which case Wilcoxon Signed Rank Tests were used.

Seedling Trait	t-test	Wilcoxon Signed Rank Test
Growth (ΔD^2H)	0.237	ND*
RGR	ND	0.317
%N	ND	0.586
%P	0.393	ND
%Ca	0.087	ND
N:P ratio	ND	0.598

* ND: No Data

Table 2.5. Summary of P value of effect of inoculation, AM plant proximity, and their combination on the seedling response variables.

Seedling trait	Inoculation		AM Plant Proximity		Inoc*AM Plant Proximity	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	0.1173	0.5341	-0.5555	**0.0187**	0.0085	0.9712
RGR	-0.0001	0.2877	-0.0001	0.5469	-0.0001	0.3990
%N	0.0150	0.6003	-0.1263	**0.0004**	-0.0196	0.5767
%P	0.0009	0.5317	-0.0084	**<.0001**	-0.0015	0.3979
%Ca	0.0179	0.2260	-0.0661	**0.0003**	-0.0093	0.6087
N:P ratio	0.1683	0.2743	-0.5343	**0.0051**	-0.1505	0.4255

Table 2.6. Summary of P value of effect of inoculation, pH, and their combination on the seedling response variables.

Seedling trait	Inoculation		Soil pH		Inoc*Soil pH	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	-0.0069	0.9755	2.5822	**0.0136**	0.3859	0.7100
RGR	<-.0001	0.7199	0.0009	0.2383	0.0011	0.1594
%N	0.0167	0.5571	0.5271	**<.0001**	0.0336	0.7984
%P	0.0011	0.4487	0.02634	**0.0002**	-0.0084	0.2273
%Ca	0.0193	0.2038	0.1206	0.0873	0.02234	0.7504
N:P ratio	0.1865	0.2071	3.3314	**<.0001**	-1.0473	0.1265

Table 2.7. Summary of P value of effect of inoculation, water table level in cm (WTL), and their combination on the seedling response variables

Seedling trait	Inoculation		WTL		Inoc*WTL	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	-0.0135	0.9531	-0.0233	0.4150	-0.0157	0.5809
RGR	<-.0001	0.6752	<-.0001	0.1549	<-.0001	**0.0446**
%N	0.0053	0.8505	-0.0131	**0.0003**	-0.0019	0.5886
%P	0.0009	0.5677	-0.0007	**0.0003**	<-.0001	0.8201
%Ca	0.0192	0.2122	-0.0012	0.5450	-0.0007	0.7244
N:P ratio	0.0784	0.5580	-0.0663	**<.0001**	-0.0208	0.2127

Table 2.8. Summary of P value of effect of inoculation, peat bulk density (BD), and their combination on the seedling response variables

Seedling trait	Inoculation		Soil BD		Inoc*BD	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	-0.0312	0.8905	-25.8079	0.3606	-47.0589	0.0964
RGR	<-.0001	0.6543	-0.0174	0.3900	-0.0240	0.2349
%N	0.0162	0.5854	0.5347	0.8848	-2.4095	0.5140
%P	0.0009	0.5728	-0.2952	0.1319	-0.1748	0.3715
%Ca	0.0174	0.2554	-2.5612	0.1828	0.9229	0.6305
N:P ratio	0.1736	0.2701	-5.3936	0.7849	24.7174	0.2120

Table 2.9. Summary of P value of effect of inoculation, percent of full sunlight at the seedling canopy (%light), and their combination on the seedling response variables

Seedling trait	Inoculation		%light		Inoc*%light	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	0.1327	0.4815	0.0144	**0.0148**	0.0061	0.3008
RGR	<-.0001	0.2951	<.0001	0.6836	<.0001	0.3752
%N	0.0055	0.8475	-0.0037	**<.0001**	-0.0004	0.6334
%P	0.0003	0.8217	0.0003	**<.0001**	-0.0001	0.3967
%Ca	0.0121	0.3964	-0.0024	**<.0001**	0.02234	0.7504
N:P ratio	0.1865	0.2071	3.3314	**<.0001**	0.0001	0.9749

Table 2.10. Summary of P value of effect of inoculation, first axis of PCA of mycorrhizal host type cover and basal area (AM Plant Index), and their combination on the seedling traits. Higher values of AM plant index indicate greater AM host cover and basal area.

Seedling trait	Inoculation		AM Plant Index		Inoc*AM Plant Index	
	Estimate	P	Estimate	P	Estimate	P
Growth (ΔD^2H)	0.1220	0.5206	0.1568	0.4097	-0.0116	0.9513
RGR	-0.0001	0.2903	-0.00003	0.8088	0.0001	0.3024
%N	0.0189	0.5025	0.1442	**<.0001**	0.0118	0.7246
%P	0.0012	0.3912	0.0091	**<.0001**	-0.0009	0.6052
%Ca	0.0198	0.1912	0.0381	**0.0328**	0.0141	0.4275
N:P ratio	0.1924	0.2010	0.7411	**<.0001**	0.1714	0.3324

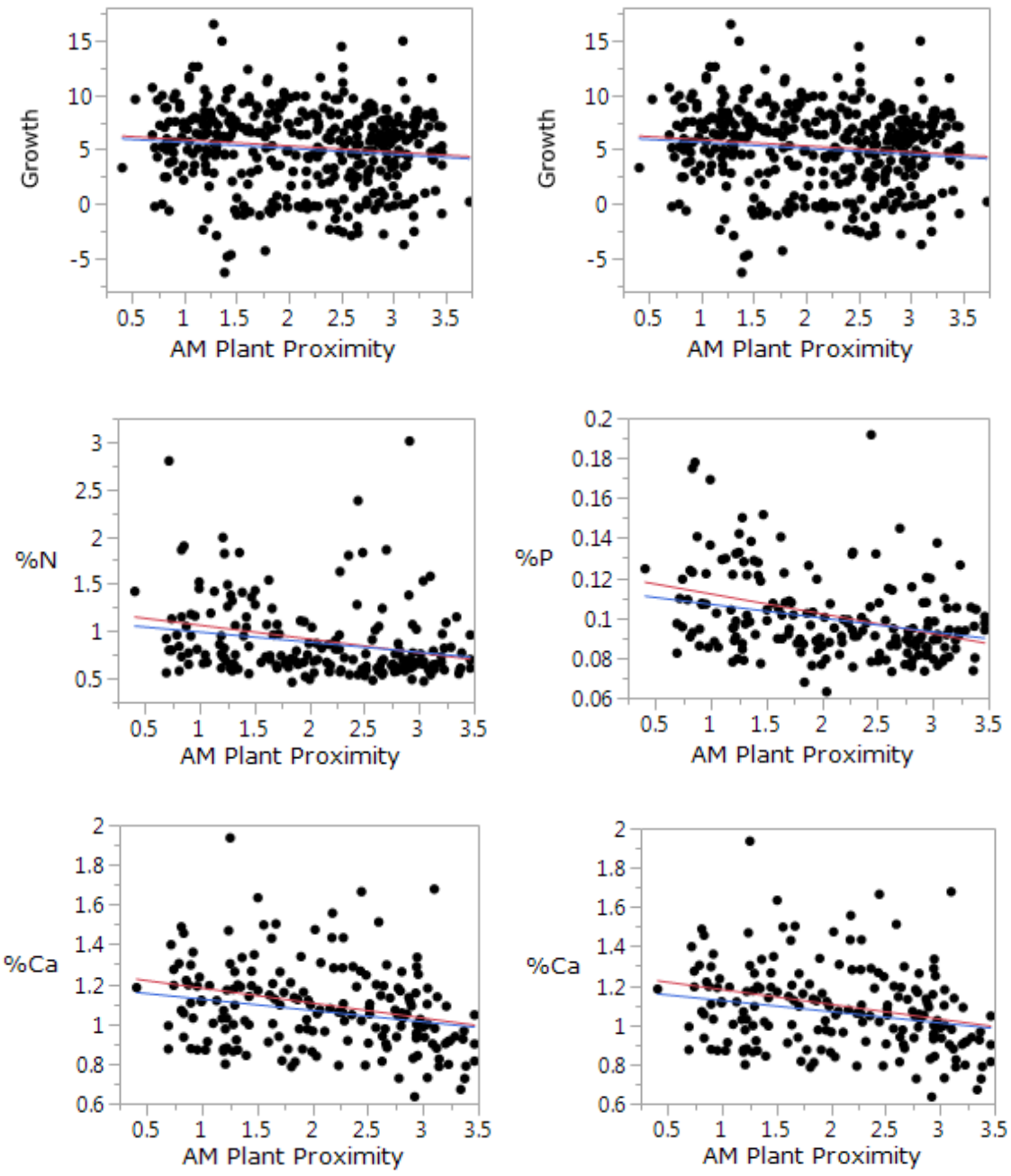

Fig 2.4. Effect of log transformed distance to the nearest AM tree (AM plant proximity) on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line)

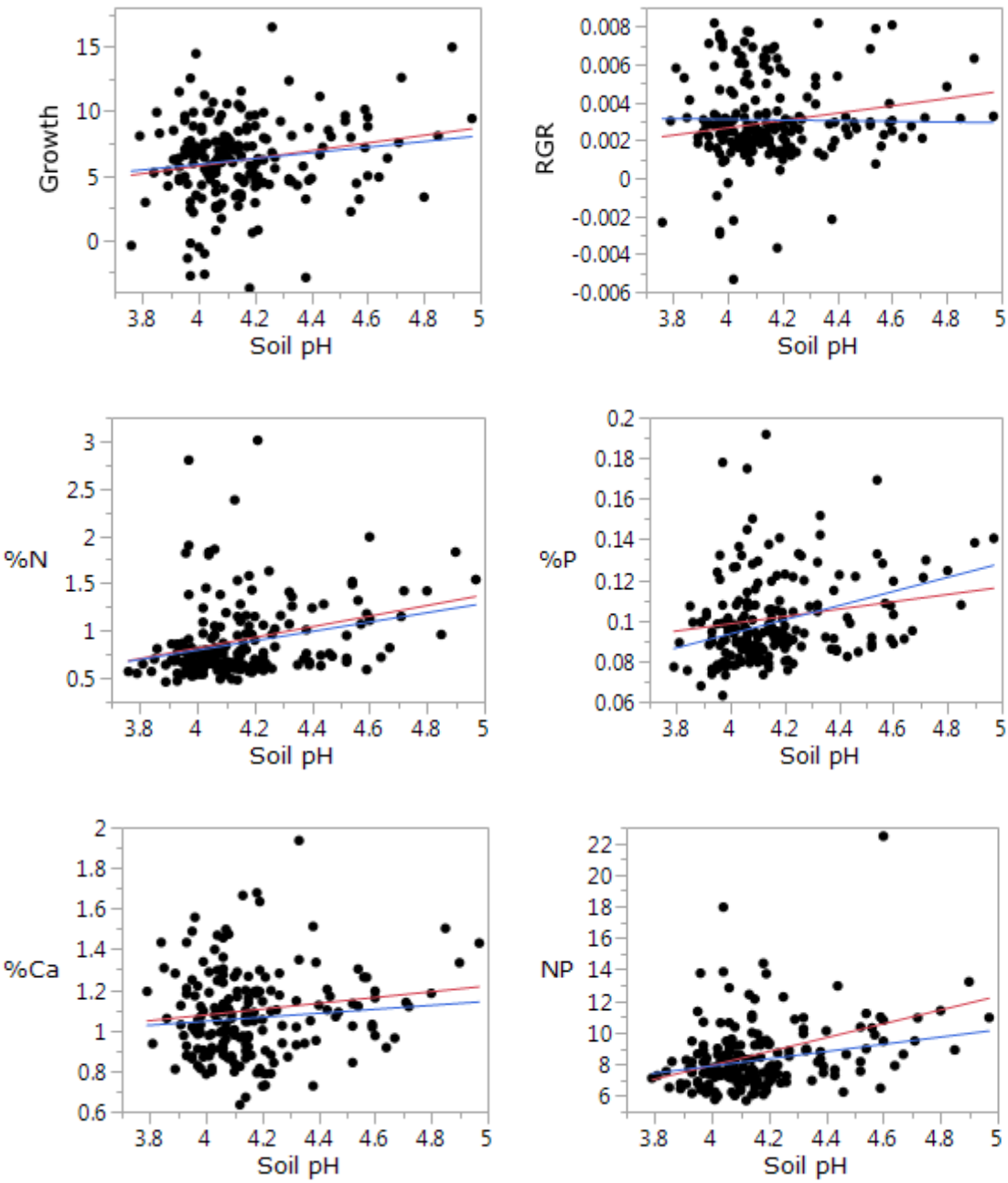

Fig 2.5. Effect of soil pH on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line).

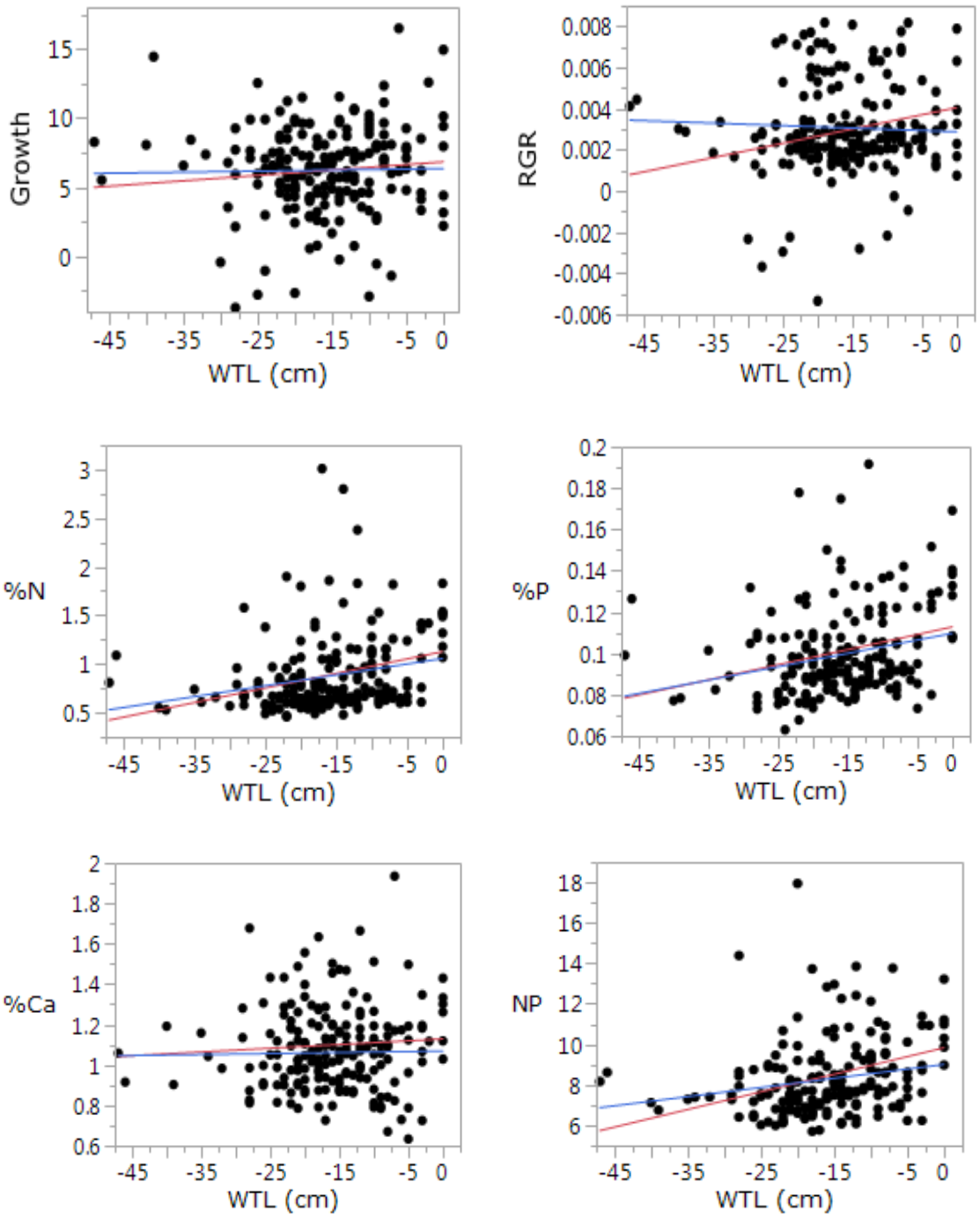

Fig 2.6. Effect of water table depth (WTD) on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line).

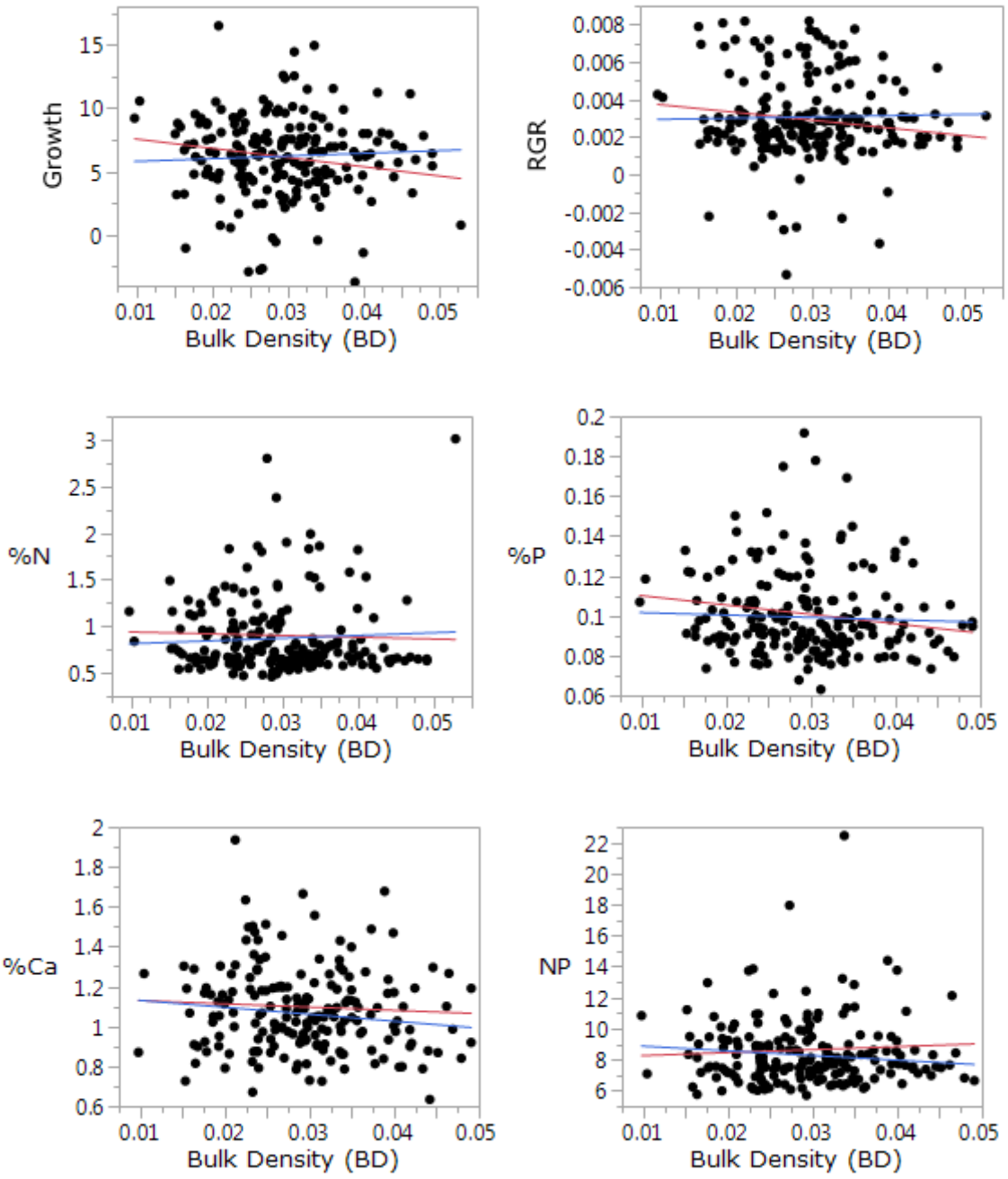

Fig 2.7. Effect of soil bulk density (BD) on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line).

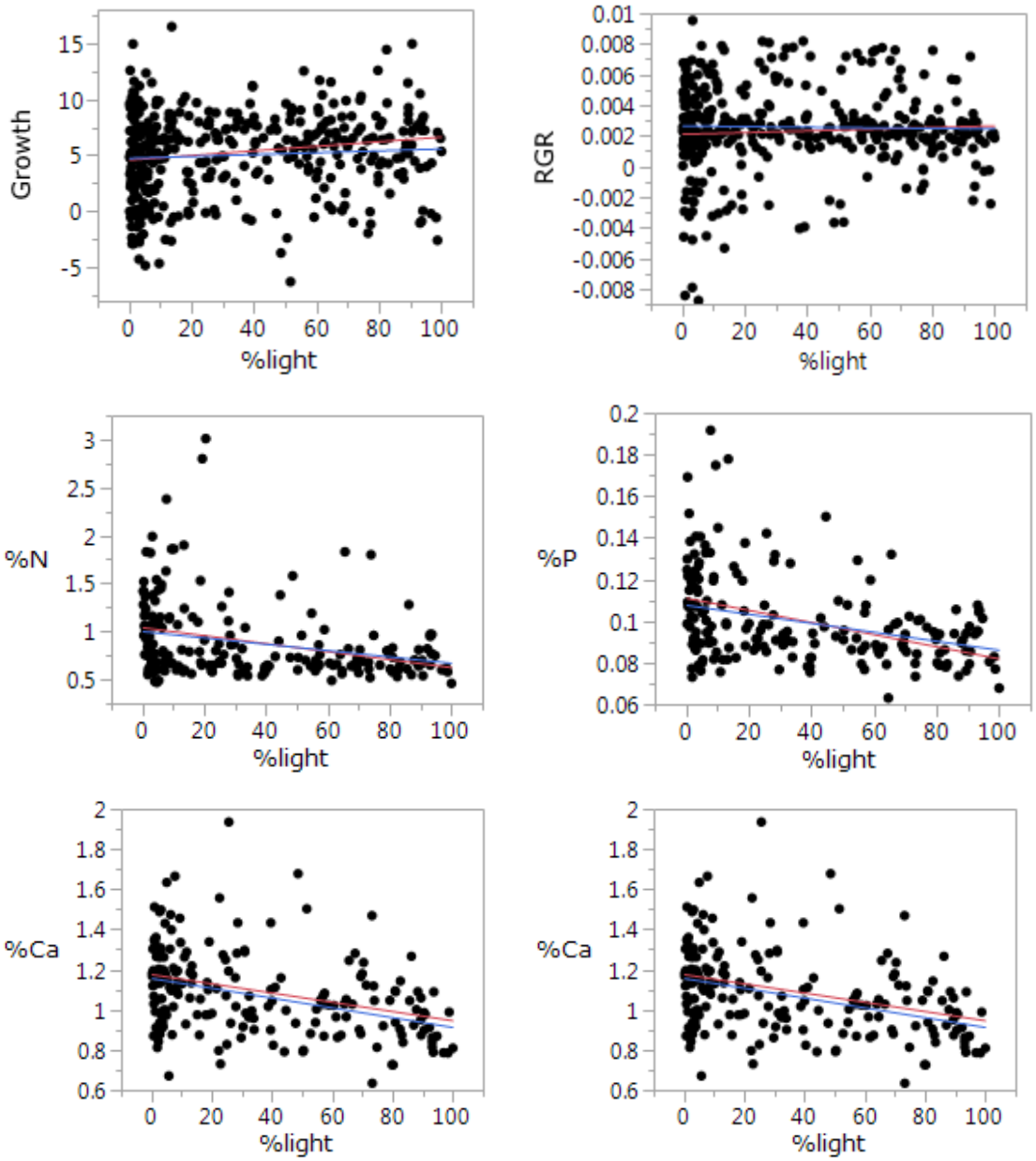

Fig 2.8. Effect of light intensity on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line).

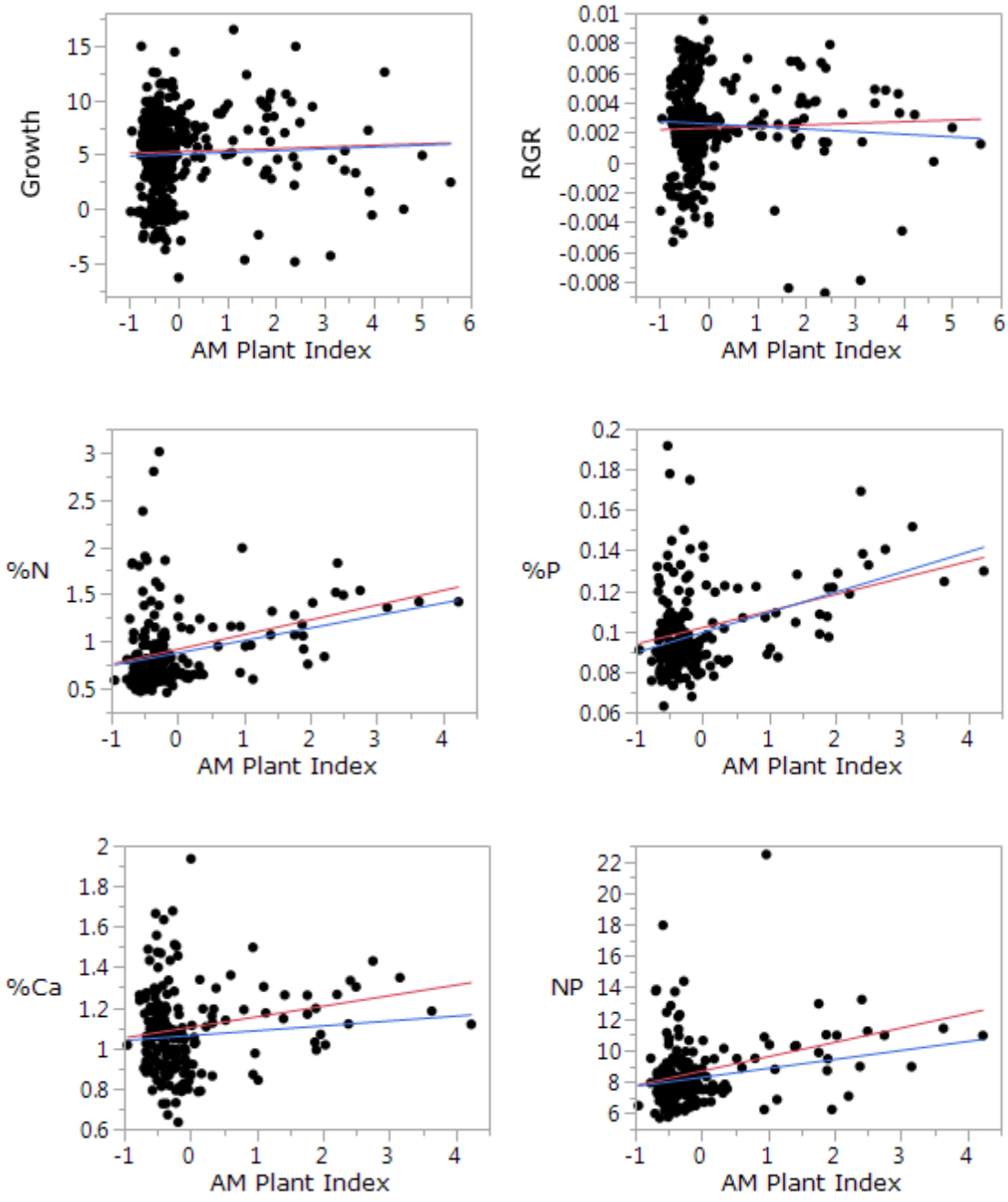

Fig 2.9. Effect of the AM plant index (first axis of the cover and basal area PCA for the different mycorrhizal types) on NWC seedling growth and nutrient acquisition (inoculated with red line and uninoculated with blue line).

Table 2.11. Relationship between distance to the nearest AM tree (AM plant proximity) and other predictors

Other Predictors	R^2	P Value
LogdistAM+1 *Soil pH	0.1982	0.1280
LogdistAM+1 *Water Table Depth	0.1982	0.2289
LogdistAM+1 *Peat Bulk Density	0.1982	0.7120
LogdistAM+1 *Light intensity	0.1982	**0.0002**
LogdistAM+1 *Ericoid Mycorrhizal Plants Cover	0.1982	**0.0002**

Table 2.12. Summary of the best multiple regression models of the effect of the suite of predictor variables on the seedling response variables

Seedling response variable	Predictors	R^2	pH	WTD	%light	AM Plant Index	AM Plant Proximity
			P	P	P	ɔ	P
Growth (ΔD^2H)	pH, WTD, %light, AM plant index[1], AM proximity[2]	0.097	0.062	0.055	**0.029**	**0.027**	0.145
RGR	pH, light	0.009	0.310		0.497		
%N	pH, WTD, %light, AM proximity	0.163	0.114	0.101	**0.003**		0.061
%P	pH, %light, AM plant index, AM proximity	0.251	0.081		**<.001**	0.104	**0.021**
%Ca	%light, AM proximity	0.168			**<.001**		0.019
NP ratio	pH, AM proximity	0.113	**<.001**				**0.025**

1 AM plant index = F1 cov/BA, first axis of the mycorrhizal type PCA

2 AM proximity = logAMdist+1, log of the distance to the nearest AM tree + 1

Appendix Table 2.1. Summary statistics for sample plots in the peatland field experiment.

Variables	Median	Average	Standard Deviation	Minimum	Maximum
Growth (D^2H/cm^3)	5.69	5.160	3.727	-6.270	16.486
RGR	0.002	0.002	0.002	-0.008	0.009
Foliar % N	0.73	0.89	0.41	0.46	3.01
Foliar % P	0.094	0.100	0.021	0.063	0.191
Foliar %Ca	1.06	1.08	0.209	0.634	1.935
N:P ratio	7.85	8.44	2.150	5.707	22.447
Soil pH	4.10	4.16	0.22	3.76	4.97
Bulk Density (BD)	0.03	0.03	0.008	0.009	0.052
%full sunlight	25.3	35.0	31.9	0.25	100
Water table level (cm)	-16	-16.08	8.27	-47	0
AM Plant Index	-0.34	0.0001	1	-0.98	5.59
AM Plant Proximity ($log_{10}m$)	2.176	2.110	0.802	0.405	3.73
ERM Cover[1]	11	10.03	3.706	0	19

[1]: data of ERM cover refer to sum of cover class of ERM (Ericoid mycorrhizal) species within $1m^2$ quadrat.

Chapter 3: Structure and composition of arbuscular mycorrhizal community on *Thuja occidentalis* roots in peatland, mesic upland, and mine tailing habitat types[3]

3.1. Abstract

Arbuscular mycorrhizal (AM) fungi are widespread symbionts mostly found in terrestrial ecosystems and some wetlands. These fungi that are composed by fungal species belongs to phylum Glomeromycota, form a mutualistic association with most land plants including northern white cedar (NWC). We assessed certain factors influencing structure and composition of AM fungi in NWC roots in three habitat types (peatlands, mining-derived stamp sands, and uplands). We hypothesized that these root-associated fungi have habitat specificity; AM fungi are a prominent component of the fungal community in all the habitats; and soil pH and plant community are significant predictors of structure and composition of Glomeromycota. We conducted a molecular study using a next generation sequencing to identify structure and composition of Glomeromycota from the three habitat types. Through a study series including root sampling from all the habitats (14 locations), processing DNA extraction, sequencing with the Illumina MiSeq, bioinformatics, and multivariate statistics, we found that Glomeromycota were a significant component of the fungal community across the habitats. Habitat type significantly affected fungal community richness. Stamp sands had the lowest richness across the habitats. Some species of these fungi were indicator species of different habitat types. Fungal community composition in stamp sand differed most from the other two habitat types.

[3] The material contained in this chapter is in preparation for submission to a journal.

Community composition was affected by soil pH for the Glomeromycota and for all fungal taxa. Likewise, %AM tree basal area strongly affected fungal community. A diverse array of unidentified dominant Glomeraceae OTUs was found in both uplands and peatlands. These Glomeraceae merit testing as inoculum for use as general and habitat-specific inoculum in NWC restoration projects in disturbed lands.

3.2. Introduction

Arbuscular mycorrhizal (AM) fungi, are formed by fungi in phylum Glomeromycota. There are currently about 250 species belong to this group (Oehl et al., 2008; Oehl et al., 2011). They occur in most terrestrial ecosystems, forming a mutualistic association with a vast majority of plants (Wang and Qiu, 2006; Smith and Read, 2008) and are also found in some wetland ecosystems (Turner et al., 2004; Ypsilantis et al., 2007; Wilde et al, 2009; Wang et al., 2010). AMF also occurs in mining soils with high concentrations of heavy metals (Turnau et al., 2001; Gildon & Tinker, 1981; Sambandan et al., 1992).

AM fungi play important roles for improving growth of plants in nutrient-poor marginal lands by mobilizing essential mineral nutrients, especially phosphorus (Smith and Read, 2008; Wang et al., 2011); metal detoxification; and reducing the effects of other plant stress factors such as drought, soil acidification, and plant pathogens (Finlay, 2008; Smith and Read, 2008). Some studies found that

Mycorrhizal fungal community function, structure, and composition are strongly affected by environmental factors (Treseder and Cross, 2006). Environmental changes might alter species composition, which can alter the diversity

and productivity of plant communities (van der Heijden et al., 2008; Chaudhary et al., 2008; Opik et al., 2010). Major factors in structuring AM communities are niche-based processes and environmental screening (Lekberg 2007; Dumbrell et al. 2010). Soil nutrient availability, soil acidity, and soil moisture strongly affect structure and composition of AMF communities (Bethlenfalvay et al., 1982; Stahl and Smith, 1984; Fitzsimons et al., 2008; Liu et al., 2009; Johnson et al.; 2010). However, role of the niche and natural processes that affect structure of fungal communities are still poorly quantified (Dumbrell et al., 2010; Klironomomos et al., 2001). It is important to study composition and distribution of Glomeromycota fungi in various ecosystems to determine factors regulating AM fungal communities.

Northern white-cedar (NWC; *Thuja occidentalis L.*) forms arbuscular mycorrhizas (Malloch and Malloch, 1985; Brundrett et al., 1989; Matthes-Sears et al., 1992; chapter 1 and 2 this dissertation). NWC commonly grows in both upland and lowland habitats. In uplands, NWC generally grows in abandoned pastures, seepage areas, limestone cliffs, and boulder fields, but grows best on mesic mineral soils with neutral or slightly alkaline soils (Johnston, 1990). In lowlands, this species predominantly grows in calcareous rich swamps. However NWC can also be found in acid peatlands, including bogs (Hannah, 2004) and poor fens (Bhatt, 1969; Scott and Murphy, 1987; Johnston, 1990; Miller, 1990; Hofmeyer et al., 2009)

In addition to basic ecological interest in NWC, this species is also an important target for ecological restoration and post-mining land reclamation. However, low soil fertility due to nutrient deficiencies, drought, accumulation of heavy metal concentrations, loss of organic matters, and loss of soil microorganisms including AM propagules become primary obstacles to successful restoration and post-mining land reclamation programs (Reeves et al., 1979; Miller and Jastrow,

1992). A number of studies have suggested application of mycorrhizal fungi to accelerate restoration and reclamation programs by reintroducing mycorrhizal propagules into the soils of their native population (Allen, 1991; Kumar et al., 2010). Mycorrhizal association with plants in the degraded lands yields numerous benefits such as plant growth improvement, mineral nutrient acquisition, pathogen protection, and metal toxicity reduction (Borowics, 2001; Al-Karaki et al., 2004).

Structure and composition of fungal communities in general, and Glomeromycota species in particular, on NWC roots have been poorly studied. Hence, we conducted research with the following aims: 1) to test effect of habitat specificity on fungal species, 2) to determine major indicator fungal species of each habitat, 3) to determine diversity and similarity of fungal species in each habitat, and 4) to determine effect of soil pH and plant community as predictors of fungal community composition and structure. We assessed some factors that could be important regulators of diversity and fungal community composition and structure. We had three questions regarding fungal communities on NWC roots in three strongly contrasting environments: 1) is there habitat specificity for fungal species in general, and Glomeromycota in particular, 2) are fungal communities more similar within habitat types than between them, and 3) are soil chemistry and plant community significant predictors of fungal community composition and structure?

We hypothesized that: 1) there is root fungal habitat specificity for the contrasting habitat types in the study, 2) Glomeromycota are the predominant root fungal community in all the habitats, and 3) soil pH and neighboring plant community are significant predictors of fungal community composition and structure in roots of NWC.

3.3. Materials and methods

3.3.1. Sampling sites

We sampled AM fungus from NWC roots across 14 sites in Houghton and Keweenaw counties in the Upper Peninsula of Michigan (Table 1). All sites had a large component of NWC, but different in habitat type with six peatlands, three stamp sand, and five mesic upland sites. Sites varied in soil pH, NWC foliar chemistry, and basal area of AM and ECM trees (Table 3.2-3.3)

3.3.2. Sampling collection and analysis

In the field

From each location, we selected 6 sample points to collect NWC roots, leaves, and soils. The site location was recorded by GPS. For sites with high tree density, we chose a center point in the middle of the study area. Then, we ran a randomly located and oriented transect and chose the first six mature focal trees at each site with a minimum distance between each pair of trees of 10 m. For two of the three stamp sand sites tree density was lower so the transect method was too difficult to apply. At these sites we chose the tree and determined the distance arbitrarily. After arriving at the site we identified locations with NWC present, and then selected six mature trees with a minimum spacing of 10 m.

We selected a ground cover, root and soil sampling point at about 50 cm distance from the sample tree. At the point of soil sampling we used a 50 cm x 50 cm PVC frame to estimate percent cover of grass, herb, tree seedling, moss, litter, leaf litter. Before taking the soil sample, the shovel was cleaned of any soils. We took a sampled 25cm x 25 cm x 20 cm soil samples and put it into a 2 gallon plastic

bag to send to the lab. Then, we identified basal area and species of trees nearby the sample point by the wedge prism method (Hemery, 2011). In addition, we picked terminal section of branch with green leaves (~10 cm long) of three of the lowest branches of the target NWC tree. The leaves were put in a labeled paper bag. We then measured slopes and aspect the area by clinometer and compass.

In the lab

We dried NWC leaves in oven at 60^{o}C for 48 hours. The dry leaves were ground to a fine powder in a mortar. Foliar N and C were measured at the Soil Laboratory of School of Forest Resources and Environmental Science, Michigan Tech using a Costech 4010 Elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA) calibrated with atropine. Foliar P and Ca were determined using the dry ash method, on a Perkin Elmer Optima 7000DV ICP-OES (PerkinElmer Inc., Waltham, MA, USA).

All the soil samples were stored at 4^{o}C when we arrived in the lab. Within 24 hours we picked the fine root samples from the soil samples, gently washed them on a sieve using tap water, and selected healthy young fine NWC roots (easily identified by their distinct morphology, paler color and turgidity) from other roots. These roots were frozen in a -20°C freezer to await DNA extraction, and the soil samples were air dried at room temperature.

The soil samples were analyzed for pH and nutrient content. To measure pH, we used a pH meter (Denver Instrument Model 220, Denver Instrument, Arvada, CO, USA) with soils rewetted with a mass ratio of 1(dry soil):40 (DI H_2O). This ratio was chosen to accommodate the peat soils.

To prepare DNA extract, the frozen root samples were freeze dried overnight using a Labconco Freeze Dry System/Free Zone 4.5 (Labconco, Kansas City, MO, USA). To be certain of low final moisture content, the samples were dried several days. The dried samples were stored in closed tubes in a sterilized desiccator cabinet. We took 0.03 g dry wt. subsamples, ground them to a fine powder in a mortar and pestle under liquid nitrogen, and put into the labeled vials.

The root DNA was extracted using the PowerSoil DNA Isolation kit following manufactured protocol (MoBio Laboratories Inc., Carslbad, CA). The DNA extract was quantified with a Qubit Fluorometer (Thermo Fisher Scientific Inc, Grand Island, NY). PCR was carried out on these samples using bar-code tagged primers appropriate for arbuscular mycorrhizal fungi. We used the forward primer 5.8SLT1 (5′ to 3′ = AACTTTYRRCAAYGGATCWCT) and reverse primer ITS4mod_long (5′ to 3′ = AGCCTCCGCTTATTGATATGCTTAART) designed to amplify the second fungal Internal Transcribed Spacer (ITS2) region (D.L. Taylor, in prep). The samples were processed at Northern Arizona University Environmental Genetics and Genomics Laboratory (EnGGEN; http://www.nau.edu/Merriam-Powell/EnGGen/), where DNA extracts was subjected to a 1:1 bead cleanup modified from Rohland and Reich (2012). The samples were normalized to 2ng/ul, and dual indexed amplicon libraries were generated with the primers 5.8SLT1 and ITS4mod_long where each end of the amplified fragment contained unique 8 bp Golay barcodes, primer pads, primer linkers, and Illumina adaptors. Paired end sequencing (250 x 250bp) was conducted on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). Mi-Seq sequences were subjected to bioinformatics and statistical analysis using the QIIME pipeline.

3.3.3. Bioinformatics

Sequence data processing began with removal of PhiX sequences from raw fastq sequence files (both forward and reverse reads) using the PhiX filtering workflow in *akutils* (https://github.com/alk224/akutils). Adaptor and primer artifacts were checked and removed using manual *grep* searches, as well as *Fastq-mcf* in *eautils* (https://code.google.com/p/ea-utils/). Next, dual indexed barcodes contained in separate barcode fastq files were concatenated to create 16bp barcodes in a single file with the *concatenate_fastqs.sh* script in *akutils*. Qiime 1.9 (Caporaso *et al*. 2010) was used to join paired-end reads with a minimum difference of 30 percent and a minimum 30 bp overlap. This was followed by demultiplexing and quality filtering in Qiime 1.9 with a minimum quality score of 20, a maximum of two consecutive low quality scores prior to sequence truncation and a minimum of 95 percent of the original sequence length required for retention of truncated sequences. The ITS2 region was extracted from demultiplexed sequences using ITSx (Bengtsson-Palme et al. 2013), in order to remove conserved flanking sequences of the 5.8S and large ribosomal subunit (LSU). ITS2 sequences were then subjected to reference-based chimera detection and filtering using Uchime (Edgar et al. 2011) coupled with the UNITE 7 ITS2 chimera detection database (Nilsson et al. 2015), and clustered in to operational taxonomic units (OTUs) at the 95 percent sequence similarity level with USEARCH (CITATION). UCHIME and USEARCH were implemented in Qiime 1.9. In Qiime 1.9, taxonomy was assigned to representative sequences for each OTU with the ribosomal database project (RDP) classifier (see Porras-Alfaro et al. 2014 for implementation of the RDP classifier with the fungal ITS) trained with the UNITE 7 species hypothesis dynamic clustering dataset (released 02 March 2015; https://unite.ut.ee/repository.php; Kõljalg et al. 2013) supplemented with additional

ITS sequences from non-fungal eukaryotic lineages obtained from the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov). OTUs unclassifiable as fungi were removed from the data set. Furthermore, OTUs whose taxonomic designations were only resolved to fungal phylum were subject to manual BLAST searches in the NCBI nucleotide database and removed if there was not convincing evidence that they were fungi. The modestly conservative approach of Schmidt al. (2013) was adopted, where OTUs represented by less than 10 sequences in the entire data set were removed to filter potential sources of sequencing or clustering error. In order to avoid biases arising from differences in sequence number per sample, each sample was rarefied to 500 sequences prior to statistical analyses.

3.3.4. Statistical analysis

To test the effect of habitat on fungal community similarity, the OTU x sample matrix was analyzed using PERMANOVA with Bray-Cutis dissimilarity. To visualize the patterns of community similarity, ordination of the communities was performed with non-metric multidimensional scaling (NMDS) with Bray Cutis dissimilarity using the fourth root transformed OTU matrix. Environmental variables were correlated against the ordination axes. Both analyses were performed in Primer 6.15 (PRIMER-E, Plymouth, UK).

Indicator species were determined using R.3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Then, to test effect of habitat, soil pH, and plant community on rarefied OTU richness (S; number of unique OTUs per rarefied sample) and Pielou's evenness (J; a measure of evenness of relative abundances of OTUs—higher with few high-abundance taxa), we used JMP 12 (SAS Institute Inc.,

Cary, NC) with standard least squares regression and post-hoc pair wise comparison Tukey test.

3.4. Results

After clustering and chimera filtering, there were 1,982 OTUs that consisted of Ascomycota, Basidiomycota, Glomeromycota, and Zygomycota. Ascomycota and Glomeromycota were found to be the dominant groups in NWC roots from all habitat types. We focused primarily on Glomeromycota in this study since they are the only fungi that form arbuscular mycorrhizas.

Analysis of OTU richness showed that for both all fungal taxa and for Glomeromycota, habitat type significantly affected fungal community richness. Stamp sand richness was lower than peatland and upland (Fig. 3.1). There was negative effect of soil pH and plant community on fungal community richness. Meanwhile, evenness did not vary among habitat types or in response to soil pH (Fig. 3.2).

For Glomeromycota OTUs and all taxa pooled by class, the pair-wise comparison in PERMANOVA showed significant difference between peatland and stamp sand as well as between upland and stamp sand, but not between peatland and upland (Table 3.4). When all taxa were tested at the OTU level, all the site pairs showed significant difference, with the weakest difference between peatland and upland (Table 3.4).

Indicator species analysis with individual habitat found only 24 indicators of peatlands, 73 indicators of uplands, and 65 indicators of stamp sands. The analysis of paired habitat types found that the peatland and stamp sand pair had only one indicator species, the stamp sand and upland pair had only three indicator species,

whereas the peatland and upland pair had 22 indicators, consistent with the higher similarity between these two habitat types relative to stamp sands (Table 3.5).

Analysis of NMDS showed strong correlation between soil pH with fungal community composition in stamp sand both within Glomeromycota and for all taxa. %AM tree basal area had a strong correlation with fungal community composition for both Glomeromycota and all taxa (Figs 3.3; 3.4; and 3.5).

Foliar analysis showed that foliar %N was uniformly low, whereas foliar %Ca and %P was highest in stamp sands and lowest in peatlands (Figs 3.6). Stamp sand had the highest and uplands the lowest Ca:P ratio, whereas N:P ratios were low in all habitats (Fig 3.7). The soil pH reflected the foliar %Ca, with the highest pH in stamp sands and lowest in peatlands (Fig 3.8.)

3.5. Discussion

To our knowledge, this is the first study identifying root fungal communities on NWC using molecular approaches. The 13,000+ OTUs we found provide an in-depth picture of the structure and composition of the fungal communities. Although Ascomycota was the most commonly found phylum (mean was 77% of OTUs), this number might not directly reflect the absolute richness, because Illumina favors shorter sequence reads (Lindahl, personal communication), and Ascomycota have shorter ITS2 region than Glomeromycota.

Stamp sands stood out strongly from the other two habitat types in all analyses. Our richness analysis that showed uplands and peatlands had the highest richness, whereas stamp sand richness was lowest. Similarly, all PERMANOVA analyses found the fungal community differed significantly between peatlands and uplands vs. those in stamp sands. However, in all analyses the fungal community in

peatlands was not significant different from uplands, or only very weakly so. These findings were supported by analysis of indicator species with all combination of site pairs.

What are the likely causes of the strong divergence of the stamp sand community from that of the upland and peatland habitat? Stamp sands differ in many ways. Known as copper mining tailings, stamp sands have high copper content, low phosphorus, poor organic matter, coarse sandy loam texture, and high soil pH. Deficiency of numerous essential soil nutrients in stamp sands have been found to result in limited plant diversity and cover, productivity, and microbial activity (Li et al., 2014).

Our results are consistent with the hypotheses that the AM fungal community might be regulated by soil type (Schechter and Bruns, 2008) as well as ecological niches (McGonigle and Fitter, 1990; Helgason et al., 2002; Lekberg et al., 2006; Drumbell et al., 2010). Soil texture and moisture and total P have all been found to reduce AM fungal species richness (Miller et al., 1999; Lekberg 2007; Gosling et al., 2013). Soil conditions might explain the low diversity of fungal species in stamp sand where the areas have very droughty coarse sands with low nutrient levels (Li et al., 2014), while uplands had organic rich moist mineral soils and peatlands had wet organic soils. In addition, our findings showed the NWC trees on stamp sands had the highest foliar P concentrations, but the soil P is probably less available for plants due to high Ca^{+2} concentrations (Dumbrell et al., 2010).

The high pH in stamp sands contrasts with the lower pH of the uplands and peatlands. Our finding for NMDS analysis revealed a strong correlation between soil pH and fungal community composition in stamp sands either on Glomeromycota OTUs, all taxa OTUs, and all taxa pooled by class. Soil pH is a major predictor of

AM fungal community composition and their environmental niche plant community availability. Oliveira et al. (2005) found that richness of AM fungal species was reduced by very high pH of the anthropogenic sediment and its salinity.

Stamp sands contain elevated concentrations of heavy metals such as copper (Cu) (Li et al., 2014) which may have reduced the richness of the AM fungal community in the stamp sands. Diversity of AM fungal community might be negatively affected by occurrence of heavy metals (Pawloska et al., 1996; Del Val et al., 1999). Stamp sands exhibited low AM plant species abundance and richness that potentially induced low richness of AM fungal community either in all taxa or Glomeromycota.

Plant community (%AM) also had a strong correlation with fungal community, and was positively correlated with the upland and peatland habitats. Meanwhile, ectomycorrhizal plant community was positively correlated with fungal community in stamp sand areas. AM fungal communities might be influenced by proximity of individual plant species (Hausmann and Hawkes, 2009; Horn et al., 2014).

The top 20 Glomeromycota OTUs (Table 3.6-3.8) represent the large majority of AMF sequences in the present study. All belong to the order Glomerales. Thirteen of the top 20 OTUs were only classified to the family Glomeraceae (Table 3.6). These OTUs were mostly indicators of peatlands and uplands, but some were found across all habitat types. The most closely related AMF isolates from other studies occurred in acid (pH 3.2-5.5) organic and mineral soils in subalpine grassland and natural forest soils (Table 3.8; Ryzska et al., 2010; Lamarche et al., 2011). An unrelated Glomeraceae sp (OTU 11260) was found only in stamp sand. Its closest relative has been found on giant redwood roots in the mountains of California (Fahey et al. 2012). Meanwhile, Kruger et al. (2015) also found that Glomeraceae

dominated number of AMF-OTUs. Cordoba et al. (2001) and Turrini et al. (2010) suggested that Glomeraceae is ubiquitous, occurred in high ecosystem range such as arid soils, alkaline, and acid soils. This AMF group is abundantly found in sandy soils. Meanwhile, Glomus sp 1 v12_1 (OTU 87 and OTU 46) were abundant only in peatlands and uplands. The most closely related OTUs from other studies occurred in soil pH 5.78-6.20 in mountain meadows and clay – rich soils with low fertility (Boerstler et al. 2006). A Glomerales sp. (OTU 122) was the only other stamp sand indicator in the top 20. Its close relatives were found in circumneutral (pH 5.5-7.7) alpine meadow soils (Renker 2003). Overall, our findings showed composition of Glomeromycota especially AM fungal species of Glomeromycetes differ based on habitat types, perhaps mediated at least in part by soil pH.

3.5.1. Implications for use of Glomeraceae native inoculum in restoration

The main goal of our study is to understand how AM fungi benefit to plants and ecosystems particularly to recover disturbed lands. Use of AM fungi as a part of restoration strategy to support growth and survival of the plants in the impoverished nutrient sites is pivotal alternative due to multiple benefits of this fungi. Consider projects of land restoration globally widespread in huge various land types and Glomeraceae sp. are abundant and occurred across all habitat types, therefore Glomeraceae spp. potentially become a potential inoculum. High species richness of Glomeraceae is important to plant biodiversity of various habitat types since we may select Glomeraceae inoculum based on their specific plant and habitat type (soil properties).

Native inoculum of selected fungi is highly recommended for ecological, and economic reasons. Klironomos (2003) and Yao et al. (2008) reported that native

Glomeraceae boosted growth of the native plants more than introduced AM fungi. Likewise, Bois et al. (2005) reported success of native AM fungi to promote the plant performance in the reclamation project of oil sand areas. Our survey of AMF in different habitats can serve as the basis for assessing habitat generalist and habitat specific AMF in order to determine which have higher efficacy in seedling establishment, nutrition, and growth. Subsequent research phases should isolate and test Glomeromycota from these habitats for cross-habitat efficacy.

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3.7. Tables and Figures

Table 3.1. Sampling site habitat types, locations, and coordinates for the fungal community analysis

Habitat type	Sampling code	Location	GPS coordinate
Peatland	PA	Marsin	N47.181746° W88.643101°
	PB	R.T. Brown Nature Sanctuary	N47.030800° W88.72459°
	PC	Painesdale	N47.022740° W88.717250°
	PD	Black Creek	N47.318793° W88.464082°
	PE	Cy Clark Memorial	N47.450249° W88.196379°
	PF	Nara Trails	N47.105013° W88.542063°
Stamp Sand	SA	Huron Creek	N47.107894° W88.582433°
	SB	Red Ridge	N47.154466° W88.763764°
	SC	Black Creek	N47.328654° W88.464813°
Upland	UA	Swedetown Trails	N47.241292° W88.471573°
	UB	Houghton Elementary School	N47.114713° W88.577005°
	UC	Tech Trails	N47.105220° W88.541831°
	UD	Black Creek	N47.319236° W88.465059°
	UE	Cy Clark Memorial	N47.449980° W88.198031°

Table 3.2. Plot averages of NWC tree size, slope, and soil pH

Habitat Type	Sampling Code	# of trees[1]	Height (m)	Diameter (cm)	Slope	Soil pH
Peatland	PA	6	6.8	11.6	1.9	5.8
	PB	6	10.9	19.8	3.8	4.1
	PC	5	2.8	5.5	0	4.4
	PD	6	6.4	11.5	0.3	6.3
	PE	4	4.1	8.9	5.0	6.0
	PF	5	7.6	15.2	6.0	4.6
Stamp Sand	SA	6	2.7	5.0	5.2	7.7
	SB	6	4.3	8.9	2.0	7.2
	SC	6	7.2	13.6	8.3	7.2
Upland	UA	5	7.0	13.1	7.6	5.5
	UB	6	7.3	8.9	8.7	5.8
	UC	3	7.7	16.2	28.7	5.7
	UD	3	6.4	23.7	35	5.2
	UE	2	4.5	9.4	10.0	4.9

[1]: number of sampling trees after sequences rarefied.

Table 3.3. Plot averages for foliar nutrient concentration of NWC foliage and % basal area of mycorrhizal types. Data are presented only for trees with fungal communities successfully sequenced

Habitat Type	Site Code	# of trees	Foliar N (%)	Foliar P (%)	Foliar Ca (%)	% ECM Basal area	% AM Basal area
Peatland	PA	6	0.87	0.086	1.51	22	71
	PB	6	1.05	0.108	1.15	49	51
	PC	5	1.10	0.098	1.21	10	90
	PD	6	0.99	0.097	1.21	29	71
	PE	4	0.62	0.071	1.34	12	88
	PF	5	1.09	0.128	0.91	38	62
Stamp sand	SA	6	0.85	0.111	2.26	56	10
	SB	6	0.99	0.123	2.08	58	42
	SC	6	ND	ND	ND	96	4
Upland	UA	5	1.03	0.111	1.19	22	78
	UB	6	0.85	0.086	1.45	20	80
	UC	3	0.93	0.099	1.14	34	66
	UD	3	0.98	0.136	1.46	58	42
	UE	2	0.85	0.114	1.09	72	28

ND: No Data (due to technical reason, there were no foliar samples for SC (Stamp Sand in Black Creek).

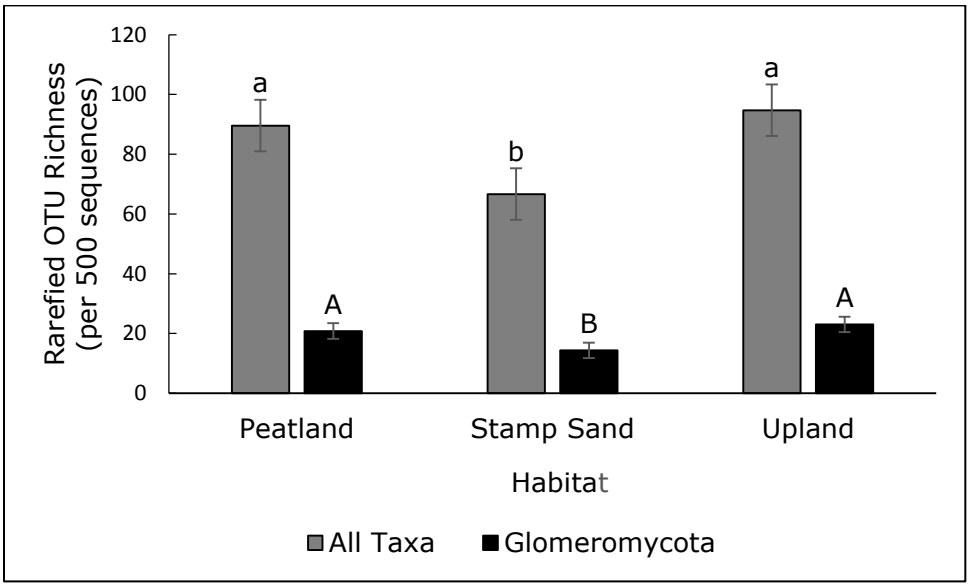

Fig 3.1. Rarefied OTU Richness of All taxa and Glomeromycota of three habitat types

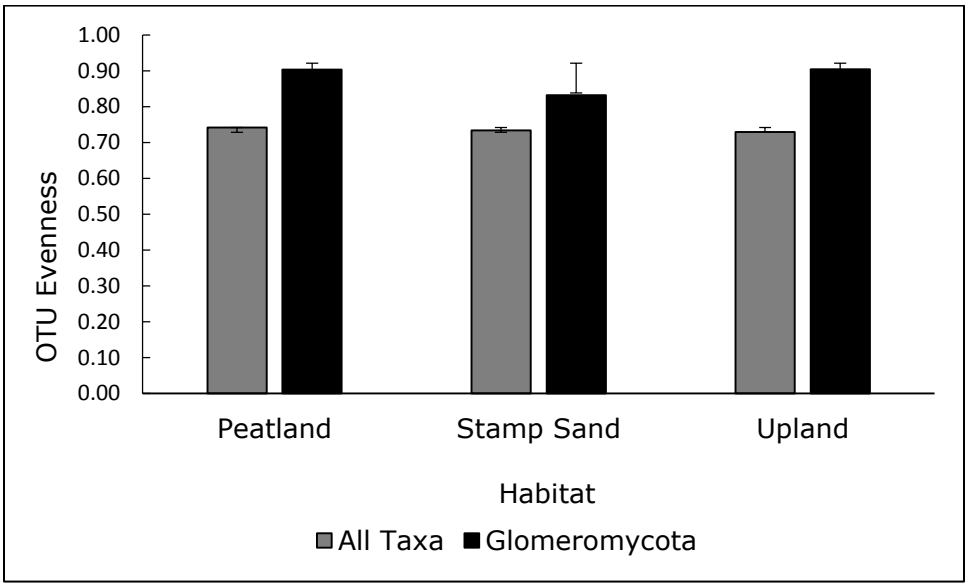

Fig 3.2. OTU evenness of all taxa and Glomeromycota of three habitat types

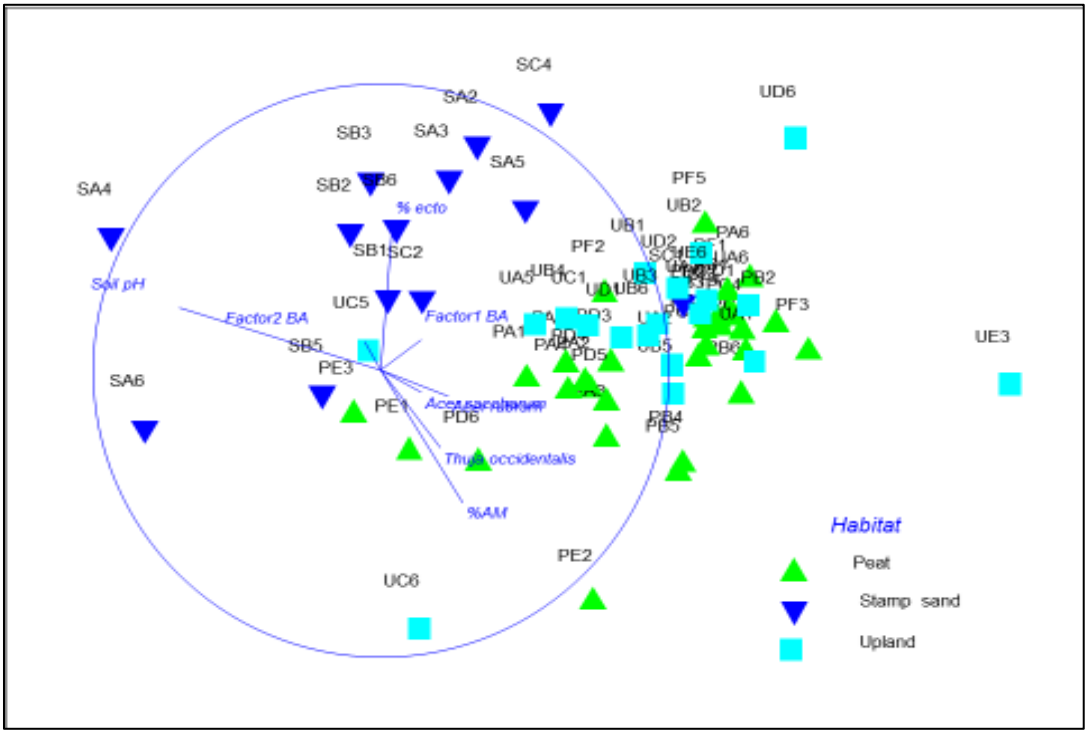

Fig 3.3. Glomeromycota OTU non-metric multidimensional scaling plot. Colored symbols represent individual samples from different sites, with individual replicates within site labelled with tree ID#. Significant correlations of predictors with NMDS axes are shown as blue lines.

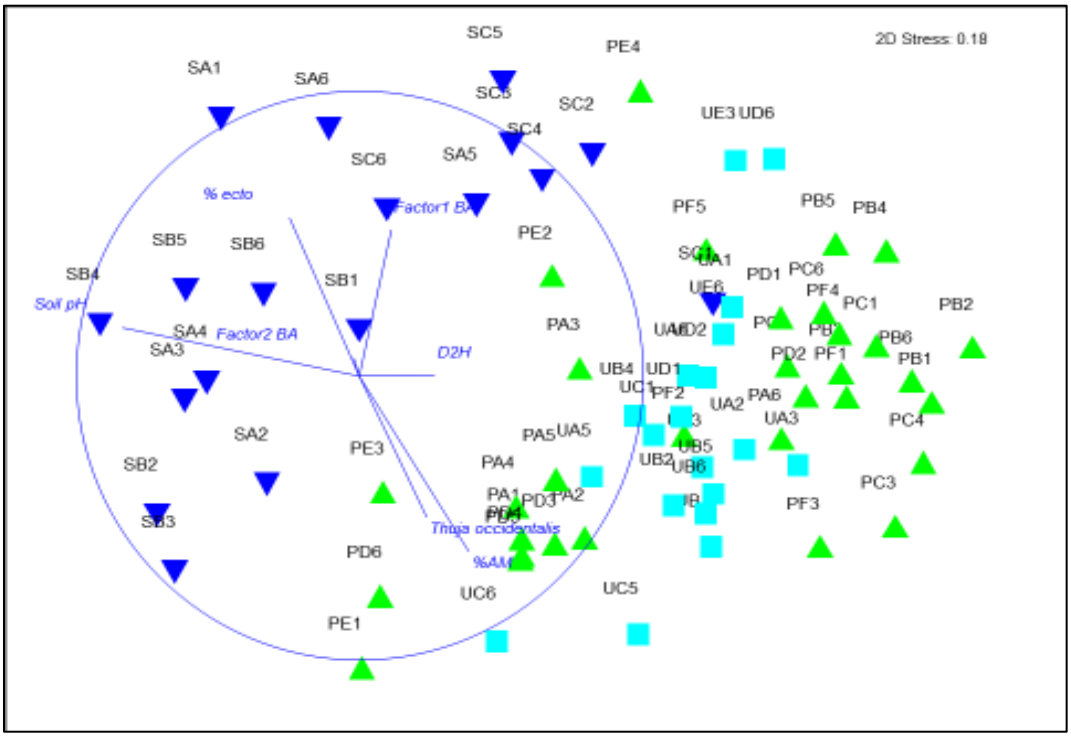

Fig 3.4. All taxa OTU non-metric multidimensional scaling plot. Colored symbols represent individual samples from different sites, with individual replicates within site labelled with tree ID#. Significant correlations of predictors with NMDS axes are shown as blue lines.

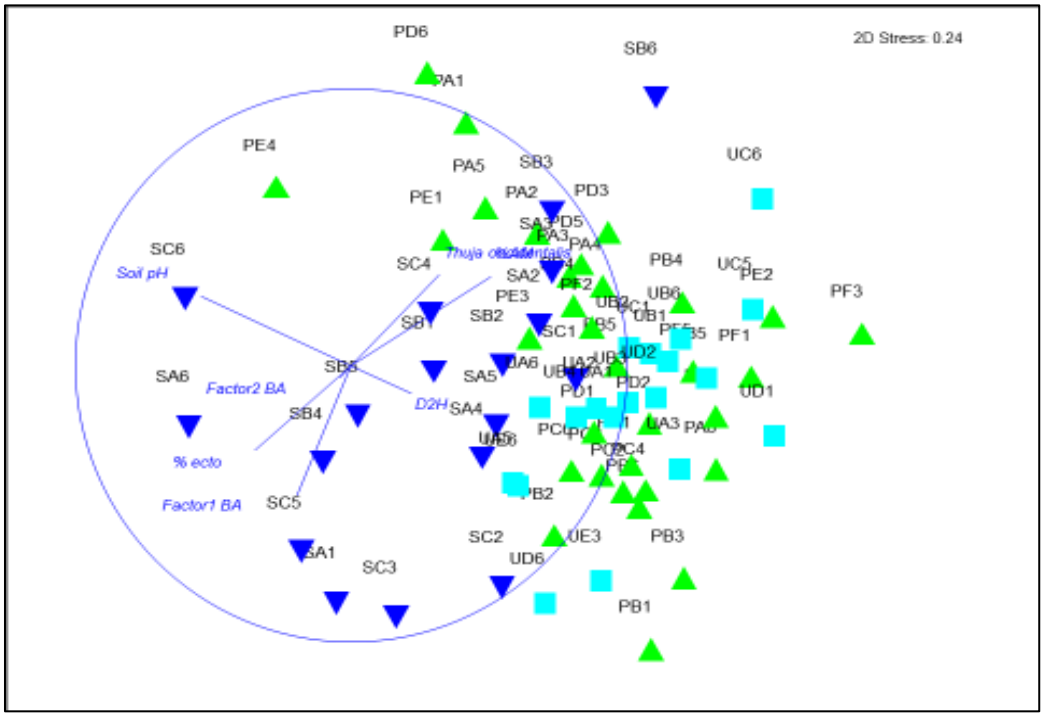

Fig 3.5. All taxa phylogenetic class non-metric multidimensional scaling plot. Colored symbols represent individual samples from different sites, with individual replicates within site labelled with tree ID#. Significant correlations of predictors with NMDS axes are shown as blue lines.

Table 3.4.PERMANOVA Pairwise test for Glomeromycota OTUs, all taxa by class, and all taxa by OTU

Groups	t	P (perm)
Glomeromycota OTU		
Peat vs Stamp Sand	1.8432	**0.0001**
Peat vs Upland	0.81484	0.8725
Stamp Sand vs Upland	1.8045	**0.0001**
All taxa Class		
Peat vs Stamp sand	1.5384	**0.0353**
Peat vs Upland	0.99767	0.4452
Stamp sand vs Upland	2.1861	**0.0003**
All taxa OTU		
Peat vs Stamp sand	1.4868	**0.0001**
Peat vs Upland	1.1734	**0.0444**
Stamp sand vs Upland	1.7582	**0.0001**

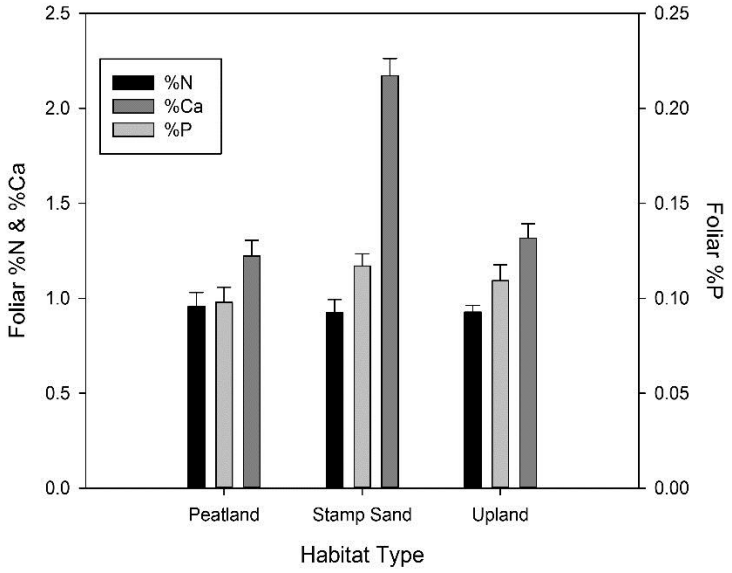

Fig 3.6. *Thuja occidentalis* foliar nutrient concentration in different habitat types. Error bars indicate SE.

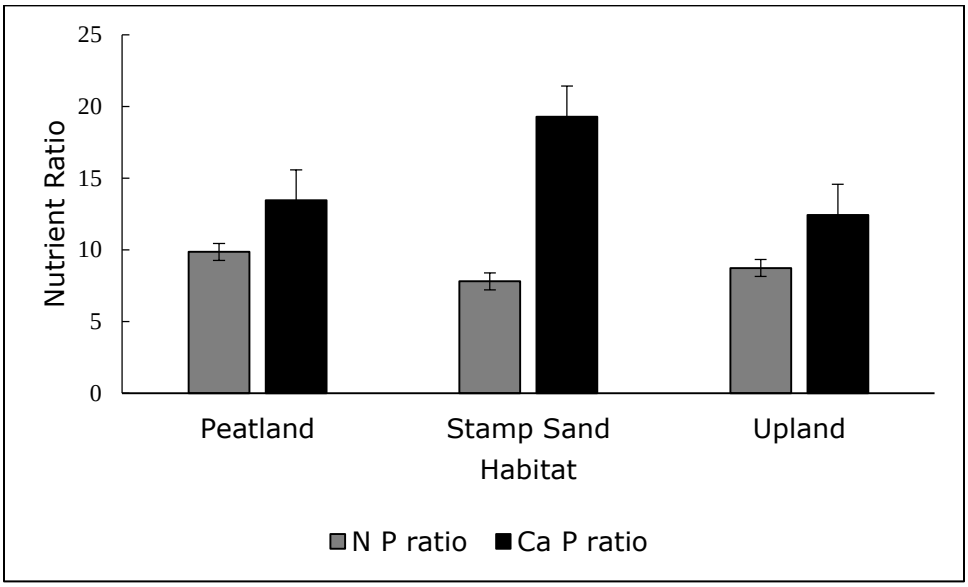

Fig 3.7. N:P and Ca:P ratios of T. *occidentalis* foliage as a function of habitat type. Error bars indicate SE.

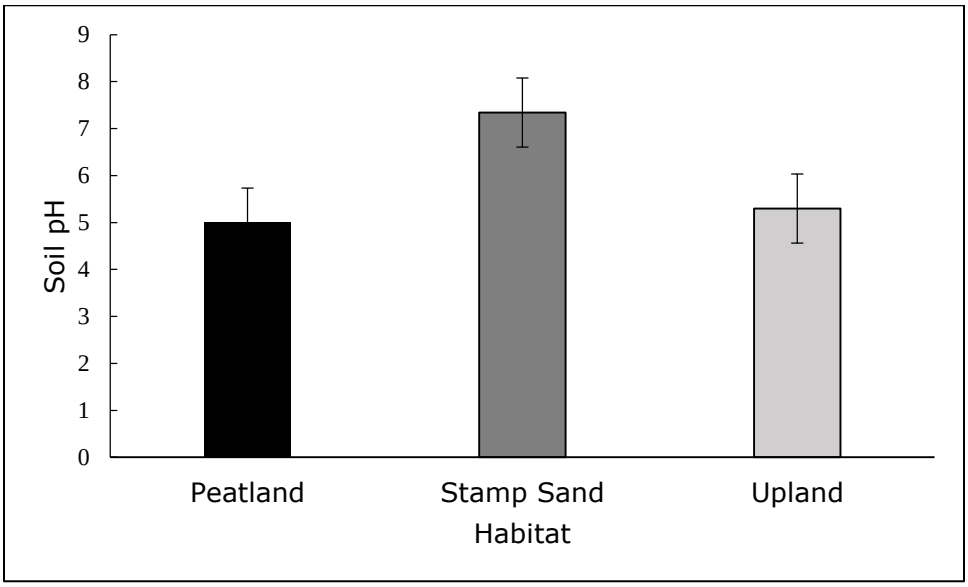

Fig 3.8. Soil pH of the habitat types. Error bars indicate SE.

Table 3.5. Fungal indicator species (up to 20 per habitat type or pair) for individual habitat types and all habitat pairs. Glomeromycota in bold.

Habitat/#OTU ID	Spesies	P value
PEATLAND	**(20 of 24)**	
9011	Helotiales	0.0032
448	*Chaetothyriales*.sp	0.006
2521	*Meliniomyces.variabilis*	0.0018
36	*Alatospora.acuminata*	0.0212
4895	*Chaetothyriales*.sp	0.01
161	**Glomeraceae**	**0.0197**
108	*Helotiales*.sp	0.0085
59	*Oidiodendron.maius*	0.0166
9751	*Xenopolyscytalum*.sp	0.0219
5	Helotiales	0.0171
229	Ascomycota	0.0334
12736	Helotiales	0.0453
3542	Helotiales	0.0159
6611	*Meliniomyces.variabilis*	0.0248
166	Helotiales	0.0498
246	Ascomycota	0.0482
7393	Ascomycota	0.0464
274	Geoglossum	0.0479
12625	Helotiales	0.0498
6850	Helotiales	0.0467
STAMP SAND	**(20 of 73)**	
6686	Helotiales	0.0001
3301	Helotiales	0.012
56	*Cenococcum*	0.0406
3835	Helotiales	0.0001
65	*Cadophora.finlandica*	0.0003
1687	*Chalara.hyalocuspica*	0.0013
106	***Glomeraceae*.sp**	**0.0005**
7093	*Cenococcum*	0.0162
10133	*Leohumicola*	0.0022
756	*Leohumicola*	0.001
10735	*Phialocephala.fortinii*	0.0032
179	***Rhizophagus*.sp**	**0.0031**
137	***Glomus*.sp.1v12_1**	**0.0098**
343	Fungi	0.0046
8680	*Cenococcum*.sp	0.0047
3248	*Chalara.hyalocuspica*	0.0047
143	Helotiales	0.0039
7137	*Rhexocercosporidium*	0.0037
359	*Geomyces.auratus*	0.0035
2912	*Alatospora*.sp.	0.004

Table 3.5 cont'd.

UPLAND	**(20 of 65)**	
1	*Meliniomyces*.sp	0.0002
12858	*Phialocephala.fortinii*	0.0002
12985	*Phialocephala.fortinii*	0.0001
3185	*Phialocephala.fortinii*	0.0022
7316	*Meliniomyces*.sp	0.0002
113	*Hysteriales*.sp	0.0002
2525	*Meliniomyces.*sp.	0.0043
107	*Meliniomyces*.sp	0.0003
6884	*Oidiodendron*	0.0006
82	*Phialocephala.fortinii*	0.0082
515	*Phialocephala.fortinii*	0.0043
2548	*Meliniomyces*.sp	0.0026
2448	*Herpotrichiellaceae*.sp.	0.0008
11254	***Glomus*.sp.7.SUN_2011**	**0.0004**
91	*Mycena*	0.0014
12150	Fungi	0.0065
2353	*Meliniomyces*.sp	0.0068
4045	*Phialocephala.fortinii*	0.0235
2513	*Meliniomyces*.sp	0.0036
614	***Glomus*.sp.1v12_1**	**0.007**
PEATLAND+STAMP SAND	**(1 of 1)**	
4	*Chalara.holubovae*	0.0156
PEATLAND+UPLAND	**(20 of 22)**	
12457	*Phialocephala.fortinii*	0.0008
12	**Glomeromycetes**	**0.0002**
22	*Cryptosporiopsis.ericae*	0.0001
16	**Glomeraceae**	**0.0001**
71	**Glomeraceae**	**0.0002**
127	**Glomeraceae**	**0.0004**
7	*Oidiodendron.maius*	0.0004
3024	**Glomeraceae**	**0.0018**
328	**Glomeraceae**	**0.0008**
147	**Glomeraceae**	**0.0021**
6185	*Meliniomyces*.sp	0.0045
11762	**Glomerales**	**0.0064**
98	**Glomeraceae**	**0.0076**
1158	**Glomeraceae**	**0.0435**
3500	**Glomeraceae**	**0.0105**
68	**Glomeraceae**	**0.0311**
2242	**Glomeraceae**	**0.0353**
148	**Glomeraceae**	**0.016**
1090	*Phialocephala.fortinii*	0.0335
197	**Glomeraceae**	**0.041**

Table 3.5 cont'd

STAMP SAND +UPLAND (3 of 3)		
1601	*Phialocephala.fortinii*	0.0039
12929	*Phialocephala.fortinii*	0.0124
11176	**Glomeraceae**	**0.0333**

Table 3.6. Top 20 most abundant Glomeromycota: abundance by habitat and indicator status. Percentages are average percent of sequence reads ± SE.

Species	#OTU ID	Peatland Percentage	Stamp sand Percentage	Upland Percentage	Indicator Status*
Glomeromycetes sp.	12	0.93±0.29	0.03±0.03	0.41±0.12	P U
Glomeraceae sp.	16	0.75±0.19	0.03±0.03	0.67±0.21	P U
Glomeraceae sp.	127	0.53±0.11	0.08±0.08	0.56±0.13	P U
Glomus sp 1v12_1	87	0.48±0.26	0.00±0.00	0.72±0.36	P U
Glomeraceae sp.	147	0.52±0.16	0.00±0.00	0.41±0.14	P U
Glomeraceae sp.	71	0.53±0.16	0.00±0.00	0.38±0.11	P U
Glomeraceae sp.	148	0.39±0.11	0.01±0.01	0.22±0.11	P U
Glomerales sp.	11762	0.36±0.10	0.00±0.00	0.28±0.13	P U
Glomerales sp.	122	0.02±0.02	0.88±0.54	0.00±0.00	S
Rhizophagus sp	58	0.11±0.05	0.59±0.28	0.11±0.06	P S U
Glomus sp 1v12_1	46	0.38±0.31	0.00±0.00	0.20±0.17	P U
Glomeraceae sp	11260	0.00±0.00	0.8±0.43	0.00±0.00	S
Glomeraceae sp.	3024	0.23±0.07	0.02±0.02	0.34±0.10	P U
Glomeraceae sp.	1229	0.21±0.07	0.08±0.08	0.28±0.14	P S U
Glomeraceae sp.	2242	0.21±0.07	0.03±0.02	0.34±0.11	P U
Glomeraceae sp.	98	0.24±0.08	0.00±0.00	0.28±0.14	P U
Glomeraceae sp.	11700	0.26±0.07	0.10±0.10	0.15±0.05	P S U
Glomeraceae sp.	695	0.2±0.08	0.10±0.10	0.22±0.08	P S U
Glomeraceae sp.	635	0.2±0.06	0.11±0.11	0.17±0.07	P S U
Rhizophagus sp	1044	0.36±0.22	0.00±0.00	0.00±0.00	P

* Indicator Status (P: Peatland; S: Stamp Sand; U: Upland)

Table 3.7. Top 20 most abundant Glomeromycota: abundance by Query coverage, E value, % Identity, and Genbank Accession # of closest match in Genbank.

Species	#OTU ID	Query Cover	E Value	Ident	Accession
Glomeromycetes sp.	12	100%	2.00E-82	98%	EU747843.1
Glomeraceae sp.	16	100%	4.00E-95	99%	EU690493.1
Glomeraceae sp.	127	98%	5.00E-74	96%	EU690493.1
Glomus sp 1v12_1	87	100%	2.00E-87	97%	AJ567795.1
Glomeraceae sp.	147	100%	1.00E-89	98%	EU747843.1
Glomeraceae sp.	71	100%	5.00E-79	95%	EU747844.1
Glomeraceae sp.	148	100%	3.00E-81	98%	EU690493.1
Glomerales sp.	11762	98%	1.00E-65	93%	EU690493.1
Glomerales sp.	122	100%	2.00E-89	97%	AJ504646.1
Rhizophagus sp	58	100%	9.00E-97	99%	EF619695.1
Glomus sp 1v12_1	46	100%	2.00E-83	97%	HQ895790.2
Glomeraceae sp	11260	100%	1.00E-84	97%	HQ895816.2
Glomeraceae sp.	3024	100%	1.00E-70	95%	EU690493.1
Glomeraceae sp.	1229	100%	1.00E-70	95%	EU690493.1
Glomeraceae sp.	2242	100%	5.00E-84	96%	EU747843.1
Glomeraceae sp.	98	100%	7.00E-93	99%	EU690493.1
Glomeraceae sp.	11700	99%	1.00E-80	95%	EU690493.1
Glomeraceae sp.	695	99%	8.00E-72	95%	EU690493.1
Glomeraceae sp.	635	100%	7.00E-78	94%	EU690493.1
Rhizophagus sp	1044	100%	4.00E-90	97%	KF836932.1

Table 3.8. Top 20 most abundant Glomeromycota: abundance by habitat association of closest match in Genbank.

Species	OTU ID	Habitat description	Soil pH	Authors & Citation*
Glomeromycetes sp.	12	Acidophilous subalpine grassland	4.8-5.5	1
Glomeraceae sp.	16	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	127	Natural forest soil	3.2-3.7	2
Glomus sp 1v12_1	87	Mountain meadows	5.78-6.20	3
Glomeraceae sp.	147	Acidophilous subalpine grassland	4.8-5.5	1
Glomeraceae sp.	71	Acidophilous subalpine grassland	4.8-5.5	1
Glomeraceae sp.	148	Natural forest soil	3.2-3.7	2
Glomerales sp.	11762	Natural forest soil	3.2-3.7	2
Glomerales sp.	122	Grassland, mountain meadows	5.5-7.7	4
Rhizophagus sp	58	Clay-rich, low-fertility Ultic Alfisols	5.75	5
Glomus sp 1v12_1	46	Soils shallow to greater < 2 m deep	ND	6
Glomeraceae sp	11260	Soils shallow to greater < 2 m deep	ND	6
Glomeraceae sp.	3024	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	1229	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	2242	Acidophilous subalpine grassland	4.8-5.5	1
Glomeraceae sp.	98	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	11700	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	695	Natural forest soil	3.2-3.7	2
Glomeraceae sp.	635	Natural forest soil	3.2-3.7	2
Rhizophagus sp	1044	No data	ND	7

* Authors & Citation: 1) Ryszka et al., 2010; 2) Lamarche et al., 2011; 3) Boerstler,B. et al., 2006; 4) Renker, 2003; 5) Parrent and Vilgalys, 2007; 6) Fahey et al,. 2012; 7) Zhang, N et al. Abuscular mycorrhizal fungi in the grasslands of northern China (unpublished).